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FOREWORD

IN THE last few years scientific papers resulting from the activities of the universities, the National Research Council and other research agencies in Canada, have become so numerous as to cause difficulty in securing prompt publication. This is largely due to the fact that there has been in Canada no national periodical devoted to research. Canadians have consequently been largely dependent upon foreign journals, already overcrowded as a result of the activities of their own scientific men. It has therefore been decided to publish, under the auspices of the National Research Council, a Canadian Journal of Research.

While the Journal is intended primarily for the publication of the results of work carried out under the auspices of the Council, it will also be open for suitable papers from Canadian research workers not connected with this organization. Material is already in sight to guarantee its publication bi-monthly. Moreover, it is expected that within a short period of time there will be available a volume of scientific papers sufficient for monthly issues. In its future development the Canadian Journal of Research can be depended upon to keep pace with the progress of scientific and industrial research throughout Canada, and the Council hopes that it may make a material contribution towards that progress.

H. M. TORY

President, National Research Council of Canada

OTTAWA, Canada, May 3, 1929.

STUDIES ON THE NATURE OF RUST RESISTANCE IN WHEAT[1]

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Abstract

A program of investigations is outlined and the results of the first three years' work reported. Eight wheat varieties, differing widely in resistance to stem rust, showed no corresponding differences in the physico-chemical properties of their expressed tissue-fluids. The infection of susceptible varieties was in some cases reduced by administering extracts of resistant varieties in petri-dish cultures or by direct injection into inoculated leaves. The injection of the juice of infected leaves into healthy leaves failed to demonstrate the presence of any toxin excreted by the fungus. The injection of salicylic acid, catechol or vanillin in suitable concentrations frequently caused a reduction in infection. These phenolic compounds in very low concentrations stimulated the growth of *Helminthosporium sativum*, but at higher concentrations inhibited it. The same phenols inhibited the germination of rust spores. On filtered wheat-juice rust spores also failed to germinate, though on unfiltered juice they germinated normally.

I. GENERAL INTRODUCTION

The Problem

Studies on the nature of resistance and susceptibility to disease in plants have lagged far behind studies on the fungi causing plant diseases and their pathological effects. The latter kind of investigation has been invaluable in pointing the way to the devising of remedial measures in many cases. There are, however, many destructive diseases for which pathological investigations have not so far suggested convenient or effective remedies. In such cases the breeding of resistant crop varieties seems to present the best hope of solving the difficulty. But here the plant breeder is handicapped by our scant knowledge of the nature of resistance to disease, a state of things which forces him to rely almost entirely on empirical methods.

The problem of disease resistance is of course of great scientific interest as well as of practical importance. During recent years a considerable number of investigators have concerned themselves with it, and some progress has been made. A great deal more work must be done, however, if the real nature of susceptibility and immunity is to be elucidated, and plant breeders placed in a position to proceed most directly to secure the factors desired. Furthermore, it is possible that studies on the physiology of parasitism may show the way to cultural and manuring practices, which, even though not conferring immunity on the crops attacked, may at least reduce the severity of the infection.

Wheat stem rust, caused by *Puccinia graminis tritici* Erikss. and Henn., is an example of a disease which it appears may be overcome most effectively by such methods.

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Contribution from the laboratories of the University of Alberta, Edmonton, Alberta, Canada, with financial assistance from the National Research Council of Canada. These studies form part of a co-operative attack on the problem of cereal rusts in Canada, carried on jointly by the National Research Council, the Federal Department of Agriculture and the Universities of Alberta, Manitoba and Saskatchewan. The present paper is the first of a series.

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Review

The literature bearing directly or indirectly on the physiology of parasitism, and the nature of resistance or immunity to disease in plants, has already become very extensive. Reviews are available which have been published quite recently (7, 11, 29, 66, 78), so that citations here will be limited to the number required to illustrate the distinctive aspects of the problem.

Blackman (7) states that we find no instance in plants of acquired immunity such as is common in animals, consequently there is no field for the development of a system of serum therapy. While this conclusion may require slight modification in the light of recent investigations (16, 19, 43, 79), it remains true that we do not usually find a pronounced general reaction in plants, such as is brought about in the animal body by the circulatory system, but are concerned rather with the relation of the parasite to the individual host cell, or to a small group of cells.

In fungous diseases of plants there appear to be two main groups of factors which may control or modify infection, namely, mechanical or structural factors, and biochemical or physiological factors. Probably the former group is of more importance in the entrance of the parasite into the host, and the latter in the establishment of nutritive relations. Such a generalization does not of course imply a necessary sequence in the incidence of these two groups of factors. Plant exudates, for example, may affect the germination of spores, thus bringing to bear a biochemical influence on the earliest stage of infection; while structural features may retard or prevent the spread of the disease within the host after infection is established.

Mechanical or Structural Resistance

The important investigations carried out in V. H. Blackman's laboratory (8, 9, 13, 20, 71) have shown that the mode of infection by *Botrytis cinerea*, *Colletotrichum Lindemuthianum*, *Sclerotinia Libertiana*, and the sporidial form of *Puccinia graminis*, is by mechanical puncture of the cuticle. In none of these instances could there be detected any chemical or enzymatic action before penetration by the infection hyphae had taken place. Cuticle is not acted upon by any known enzyme, and apparently does not permit the diffusion of enzymes or toxic products produced by these organisms. To the question why these fungi should bore through the cuticle, rather than follow the easy path of the stomatal openings as is done by the germ tubes of the urediniospores and aeciospores of the rust fungus, there is no answer yet. This mechanical mode of penetration suggests of course a possible mechanical basis of resistance, should we have tissue sufficiently heavily armoured so that it could not be punctured. Reported examples of this sort of resistance are found in connection with brown rot of plums (64, 77), and potato scab (41).

In the cereal rusts the thickness of the cuticle does not come in question, since the germ tubes enter by the stomatal openings. Here, however, there is a possibility of a closely related type of protection, which may result from the

small size of stomata, or their regulation by the plant, excluding the fungal hyphae. Miss Allen (1, 2) suggested this as a factor in the resistance of Kanred wheat.

Waxy coatings may afford protection by increasing the difficulty of wetting the surface, the latter being a prerequisite condition for the germination of spores. An example of this is found in the rust resistance of certain varieties of barley (29).

Structural features may also prevent the spread of infection following penetration. Hawkins and Harvey (27) found the hyphae of *Pythium de Baryanum* growing in potato tubers pass from cell to cell by mechanical pressure. The resistance of the tissue to puncture, its content of crude fibre, and resistance to this disease were correlated. Another example is found in varieties of flax resistant to wilt. These are stimulated by the causal organism, *Fusarium Lini*, to abundant cork formation, which bars the progress of the fungus (7).

In the brown rot of plums, caused by *Sclerotinia cinerea*, the fungal hyphae, according to Valleau (64) do not enter the host cells, so that the relationship here must be controlled largely by the structural features of the host. This author found that resistance depended upon (a) a thick skin, (b) the production of parenchymatous plugs which filled the stomatal cavity, (c) the production of corky walls in the lining cells of the stomatal cavity, (d) and the firmness of the fruit after ripening. The increased susceptibility on ripening was considered to be owing to the disappearance of the middle lamella, through which the fungus in earlier stages had to penetrate by enzymic action. Willaman and his co-workers (76, 77) found by analyses of resistant and susceptible varieties of plums at various stages of maturity, that resistance was correlated with the content of crude fibre and pentosans, and that these structural materials as well as resistance tended to disapppear with advancing ripeness.

In wheat stem rust, the fungus appears to enter the stomata and penetrate the cell walls of resistant and susceptible varieties with equal facility (with certain possible exceptions cited above from Miss Allen's work.) Consequently there appears little basis for mechanical resistance to this disease. However, susceptibility varies widely in degree, and may possibly be influenced by structural variations. Hursh (34) found that the growth of the fungus is confined to the chlorenchymatous collenchyma, and wheat varieties differ considerably in the amount and isolation of this tissue, a distribution which may be influenced by cultural and manuring methods. Stakman and Aamodt (62) concluded that while true physiologic susceptibility could not be changed by the use of fertilizers, morphologic susceptibility may be changed slightly. This conclusion is supported by the results obtained by Weiss (72) on the effects of various salts used in cultures.

Biochemical or Physiological Resistance

The composition of the infection drop in which the spores germinate on the surface of host tissues may be affected favourably or unfavourably to the parasite by diffusion of substances from the host. Brown (14, 15) found

that drops of distilled water on the leaves of a number of plants gradually increased in conductivity. The substances which diffused out of the plant tissues in many cases stimulated the germination of *Botrytis* spores placed in the drops. In some cases there was no apparent effect, and in other cases germination was inhibited. The presence of the spores, however, had no effect on the rate of exosmosis. Further tests showed the substances concerned to be volatile, and that similar effects were produced by simple chemical substances, such as ethyl acetate. In the same way, Noble (51) found that various plant distillates as well as simple chemical substances stimulated the germination of the spores of *Urocystis tritici*.

Here then we have indicated a natural chemical basis upon which plants may encourage or repel fungous attacks at the outset, subject it is true to mechanical modification by the permeability of the cuticle to the effective substances. Artificial protection of a similar kind is commonly given by various spraying and dusting treatments.

In the instances noted above, the fungi themselves have not been found to assist their entrance into the host by chemical secretions. Miss Allen (2, 3) observed, on the other hand, the rather curious fact that some forms of wheat stem rust apparently hindered their own entrance through the stomata by the excretion of some substance acting injuriously on the guard cells. The possibility was suggested that this appressorial secretion is a relic of an earlier period when the fungus secreted enough of this substance to dissolve its way into the host.

After the fungus has effected an entry into the host, we have to concern ourselves with the effects of the metabolic products of both host and parasite.

An extract of the germ tubes of *Botrytis cinerea* was found by Brown (12) to destroy the tissue by dissolving the middle lamella of the cell walls and also by direct action on the protoplasmic membrane. The extract contained an enzyme associated with a toxin. On the other hand the juices of certain plants greatly retarded the action, suggesting a chemical basis of resistance in such plants. Similarly, Reynolds (59) found that extracts of flax plants resistant to *Fusarium Lini* depressed the growth rate in cultures of this organism more than did extracts of susceptible plants.

Various chemical substances occurring in plants are toxic to fungi. Tannin is a common plant product falling in this class, and Cook and Taubenhaus (18) found instances of disease resistance apparently correlated with the rate of tannin formation in the cells. However, the specificity of parasitism (e.g. in wheat rust) is not easily reconcilable with a host toxin theory, unless the toxin is released by a specific enzyme excreted by the parasite.

Plant acids would appear to have a possible significance in two directions. On the one hand, some organic acids may be utilized in the metabolism of certain fungi, and on the other, most organisms develop satisfactorily only within relatively narrow limits of acidity. Hursh (33) claimed that in regard to the urediniospore germination of two biologic forms of wheat stem rust, the one more limited in infection capabilities was also more restricted in tolerance

of extremes of hydrogen-ion concentration. Miss Hurd (30, 31), however, was unable to find any correlation between the hydrogen-ion concentration of the host fluids and rust resistance, at any stage in the growth of wheat plants. While the reaction of the tissue fluids must have some influence on the vigour of parasitic organisms growing in them, it seems unlikely that this could ever vary within wide enough limits to confer real immunity.

Brierley (10) states that potato varieties immune to the wart disease are immune under all conditions. He suggests therefore that the defensive factor is a definite chemical substance, e.g., an alkaloid or glucoside, which is an integral part of the metabolic structure of immune varieties, rather than a structural difference, which is modifiable, or an accessory substance such as tannin or mucilage, since these are present in very variable proportions. This suggestion appears applicable to most cases of physiological resistance which have been investigated, including rust resistance in cereals.

There is an alternative view, that the failure of the infection may be owing to the absence in the host cell of some substance essential in the metabolism of the parasite, or that the parasite may lack an enzyme essential in the use of some characteristic compound of the host. Willaman (75) concluded that a vitamine obtained from the host is required by *Sclerotinia cinerea*, and the failure of yeast to thrive in the absence of "bios" supplied from an external source is well known. It is not necessary, of course, to postulate that a substance so complex as a vitamine is required in all cases; a very simple substance or even a chemical element may occasionally fill the need (5). The conclusion of Wellensiek (74) in regard to the relation of maize to *Puccinia Sorghi*, that the difference between a resistant and a susceptible host was merely one of degree and might be explained by assuming that the amount of a special nutritive substance present determined the degree of susceptibility, appears to be an adaptation of this view.

There is still a third view, that the fungus itself excretes some substance fatal to the host cell, which thereupon perishes, leaving the parasite to starve. This suggestion is particularly applicable to cereal rusts: when the haustorium of the parasite penetrates the mesophyll cell of a highly resistant wheat variety, both host cell and haustorium die very promptly. Some of Miss Allen's experimental evidence (1, 2, 3) makes it seem probable that the toxin comes in this instance from the parasite. This author observed, however, that although the first cells attacked were always killed, there took place gradually an adjustment which enabled the parasite to eke out a rather enfeebled existence for a considerable period. Host cells attacked in later stages were in some cases stimulated, the cell contents becoming richer and the plastids larger, a reaction which is commonly found in susceptible varieties. More direct evidence of the excretion of a toxin by the parasite was found by Kulkarni and Mundkur (36), when the filtrate of a culture of *Fusarium* caused healthy cotton plants to wilt, and similar cases have been discussed by Hursh (35).

The three views outlined above are substantially the same as the pioneer hypotheses of Ward (68, 69, 70).

In diseases where the relation between host and parasite is of the complicated nature we find in wheat stem rust, it is probably too much to hope that the difference between resistance and susceptibility will be found to lie in some one simple factor. The relations in the two cases may be generalized by stating that they appear to depend upon a balance, or lack of balance, between the physiological processes of the host cell and the parasite. Susceptibility means that host and parasite can live together in comparative harmony, without serious disturbance of function in either, at least during the early stages of the partnership. Eventually the host may be so heavily drained that it is unable to lay up its usual reserves of food, e.g., in wheat kernels. Resistance on the other hand denotes an incompatibility between host and parasite, and in consequence a failure of the infection.

Differences in the constitution of the proteins of resistant and susceptible hosts, as a possible cause of the incompatibility of the former, have recently been investigated serologically by Nelson and Dworak (46). Flax varieties resistant to wilt fell into one serological group. Conversely, Link and Sharp (38) found the serological specificity of certain plant parasitic bacteria to be correlated with their biological specificity. This seems a promising new line of attack on the problem.

There existed some years ago a popular impression that the susceptibility of a plant might be influenced directly by its general condition of health, and that improved methods of culture might maintain the plants in a thriftier state in which they could better ward off their fungous enemies. In view, however, of the quasi-symbiotic relationship between susceptible host and parasite, it is not surprising to find that most investigations on this point have shown the reverse to be true. Stakman and Levine (63) found that adverse environmental conditions, unfavourable for wheat, were also unfavourable for the rust parasite, decreasing its virulence and spore size. Raines (57) reported a direct correlation between vigour of host and virulence of disease in a number of other cereal rusts. An apparent exception to this relationship is reported by Lee (37), who was able to prolong for several months the sound, healthy condition of sugar-cane cuttings in the soil, by a system of pruning which maintained the cuttings in an actively functioning state. Normally, when the new shoots are allowed to form independent roots, the cuttings fall an early prey to soil fungi. But it would appear that the issue is complicated here by the onset of natural necrosis, independent of parasitic attack, in tissues which have ceased to function.

Peltier (53) investigated the influence of soil temperature, soil moisture, air temperature, relative humidity, and light intensity on infection by wheat stem rust. He found that the longevity of the urediniospores, the ease of initial infection, and the vigour of development of the disease could be modified to some extent by controlling these factors; also that two biologic forms of rust showed certain differences in adaptations. In general, however, changes in growing conditions caused the vigour of host and parasite to fluctuate together, and in no instance was the characteristic type of infection changed.

While it appears, therefore, that we cannot look to environmental adaptations as a basis for true resistance or susceptibility, we must nevertheless recognize the direct relation of environment to the metabolic status of the plant, and in consequence to the probable virulence of disease attack. Henning (28) drew attention to the relation between resistance to yellow rust in wheat and the acid-sugar ratio in the plants found in certain experiments. Dickson and Holbert (21, 22) were able to modify the severity of seedling blight in corn and wheat by controlling the growing temperatures. This result was associated with the altered composition of seedlings grown at different temperatures, particularly in regard to carbohydrate distribution. Pentose substances were shown by culture experiments to be superior to hexose compounds as a source of carbon for the causal organism, and in practice those conditions which favoured the accumulation of pentosans in the seedlings also favoured the development of blight. The metabolism of susceptible and resistant host strains was significantly different, and seemed in this instance to be the determining factor in disease reaction.

The composition and metabolic balance of the cell contents may no doubt be changed by manuring methods sufficiently to have a significant relation to parasitism. Soil conditions have, too, an important influence on the development and infection capabilities of many fungi which reach the plant through the soil. By adjusting the "toxic-antitoxic" relations of chemicals added to the soil, Picado (54) was able to inhibit the development of nodules on beans without harming the bean plants The next and more difficult step is to control the composition of the cell contents themselves, whether by plant breeding or by methods of nutrition, so that fungal growth is inhibited there also.

A Program of Investigation

A program of investigations on wheat stem rust, looking to the solution of some of the questions emerging from the foregoing discussion, was laid down at the outset as a general guide for our own laboratory. Though it was and must still, after three years' work, be considered as tentative, its presentation here in summarized form may be of value in giving more definite outline to the general problem.

A. Physiology of the host. Using as a reference host series a number of wheat varieties representing a wide range of rust reactions, an attempt is being made to discover if any particular physiological behaviour is characteristically associated with a particular type of rust reaction. The study is planned to include such generally important phases of plant physiology as photosynthesis, respiration, transpiration, enzymes and nutrition, as well as special aspects which the peculiar nature of the problem or the development of the investigation may suggest. For example, the effect of environmental conditions, methods of manuring, etc., on host vigour, metabolic balance and structural development appear of importance in the light of observations already discussed.

B. Physical and chemical properties of host-cell contents. The cell contents appear to be the seat of the crucial reactions which determine the relation of the host and the invading rust parasite. Again with the object of discovering properties associated with particular rust reactions, a study is being made of the physical and chemical properties of the cell contents of our reference series of hosts. Starting-points have included colloidal properties, physico-chemical constants, and analyses for substances which may have some relation to rust resistance. It is planned to examine also the distinctive properties of such important substances as proteins and starch.

C. Inhibiting or accessory substances. The difference between a resistant and a susceptible wheat variety, with reference to any particular rust form, may lie in the presence in the first of a substance inhibiting the growth of the parasite or, in the second, an accessory substance necessary to the metabolism of the parasite. It is true that the complicated relationship between host and parasite makes it seem more probable that a physico-chemical or physiological *system* rather than a simple chemical substance controls the rust reactions, but the point is worthy of investigation. Accordingly, the tissue fluids and various extracts of the leaves of varieties with opposite rust reactions, and certain pure substances known to occur in plants, are being used in culture and injection experiments to test the possibility of modifying the infection of both resistant and susceptible varieties. Coupled with these experiments are the analytical studies referred to above, which may throw light on the nature of effective substances in the wheat plant.

D. Physiology of the parasite. Since the rust fungus is an obligate parasite, its physiology must be studied in connection with that of the host, and controlled experiments are only possible in so far as it is possible to control the state of the host plant. This may at least be considerably modified in definite directions by regulating such things as light, temperature, humidity, carbon dioxide, water, and nutrients. Furthermore, the development of a satisfactory method of direct injection of the wheat leaves has opened up a promising way of studying the effect of various nutritive or accessory substances on the development of the rust fungus in the leaf. A study by these methods, of a reference series of rust forms varying widely in infection capabilities, should yield important information and may disclose characteristic differences in their physiological adaptations.

The progress made in carrying out sections B, C and D of this program will be reported for the most part in the following pages. The analytical studies, however, will be reported in a separate paper, and section A, dealing with the physiology of the host, in still another paper. The experiments have so far touched scarcely more than the fringe of the problem and are regarded only as breaking ground upon which may be built slowly and painfully a solid structure of investigation.

II. PHYSICO-CHEMICAL PROPERTIES OF HOST-CELL CONTENTS

Biological systems are very delicately balanced, and the course of reactions may be profoundly modified by slight changes in physical conditions. It is not surprising, therefore, that physico-chemical determinations have been frequently used in investigations of plant problems. These have shown the importance of the physical properties of plant saps in four principal relations: (a) in relation to temperature, water supply, light, and other factors of the environment; (b) in relation to the function of special organs, and the position and age of the tissue; (c) in relation to the growth habit or growth form of the plant concerned; (d) in relation to parasitism, in certain cases (24, 25, 26). On the other hand, investigations of the relation between physico-chemical constants and resistance to pathogenic fungi have for the most part yielded negative results (30, 31, 32, 34, 61, 65, 73).

We had therefore no great hope of fruitful results from this part of our work, but in view of the great complexity of the general problem, it appeared advisable to explore every possible avenue of approach, and as well to lose no opportunity of adding to both the diversity and comprehensiveness of our knowledge of the wheat varieties with which we were working. Accordingly, in the summer of 1926, a study was made of the total solid content, bound water, osmotic pressure, electrical conductivity, and hydrogen-ion concentration of our eight reference hosts, namely: Little Club, Marquis, Mindum, Kubanka, Kota, Kanred, Common emmer[1], and Khapli. These varieties range from complete susceptibility in Little Club to high resistance in Khapli, to practically all rust forms.

Methods

The wheat varieties were seeded in field plots on May 4, which is about an average date of seeding spring wheats at Edmonton. Determinations were made at four dates, about two weeks apart, beginning June 9 and continuing until July 22, when the plants were well advanced towards maturity.

The manner of collecting leaf tissues and expressing the tissue fluids has been described elsewhere (48). A variation in the method of collection was, however, necessary, for while the entire seedlings were cut off in the early collections, the leaves were stripped from the plants in the later collections. All collections were made at the same hour of the day, namely 8 a.m.

The total solid content was determined refractometrically, as described by Gortner and Hoffman (23) but with a correction in the values found necessary in earlier work in our laboratory (47). Bound water as an estimate of the colloidal content of plant-tissue fluids has been discussed by Newton and Gortner (49); it is calculated from the amount by which the freezing-point depression of the press-juice to which a molar concentration of sugar has been added is in excess of the sum of that of the pure juice and that of a molar

[1] *Common emmer was used because no seed of Vernal emmer was then at hand. The latter variety was used in all later work, its rust reactions being better known.*

solution of sugar in water. Osmotic pressure, electrical conductivity and hydrogen-ion concentration were determined in the usual manner, with Beckmann thermometer, Wheatstone bridge and potentiometer respectively, highly accurate research equipment being used throughout. All determinations were carried out promptly after expressing the tissue fluids.

Results

The values obtained for the physico-chemical constants investigated are presented in Table I. The four series of determinations are reported, together with the averages for each wheat variety and the dates of the separate determinations. As eight varieties of wheat were used, and not more than two could be dealt with conveniently in one day, the work on each series of determinations extended over four days. In the table the varieties are listed in the probable order of increasing resistance to stem rust.

The results show distinct differences between varieties, but do not show that these can be consistently related to rust resistance. The fluctuations in the values at different dates appear to be mainly governed by the stage of growth and environmental conditions. The values for total solids, bound water, and osmotic pressure increase as the season advances, but whether this trend bears any relation to recent observations (55) of increasing rust resistance with age of plant, it is impossible to say. The specially marked upward swing in the bound water of Common emmer and Khapli, in the last collection, is probably in the case of the emmer an expression of drought adaptation (44) and in Khapli the result merely of its earliness and consequently more advanced stage of maturity. Kanred is an exception in the trend of its values; it behaved in the usual manner of a winter wheat seeded in the spring, failing to joint or progress towards maturity.

There seems little ground at present for assigning to the differences found any significance from the point of view of rust resistance.

TABLE I

PHYSICO-CHEMICAL CONSTANTS OF THE PRESS-JUICE OF A SERIES OF WHEATS ARRANGED IN PROBABLE ORDER OF INCREASING RESISTANCE TO STEM RUST.

	Total solids (Per cent)					Bound water (Per cent)					Osmotic pressure (Atmospheres)					Electrical conductivity ($Kx10^3$)					Hydrogen-ion concentration (pH)				
Date	June 9–12	June 21–24	July 5–8	July 19–22	Av.	June 9–12	June 21–24	July 5–8	July 19–22	Av.	June 9–12	June 21–24	July 5–8	July 19–22	Av.	June 9–12	June 21–24	July 5–8	July 19–22	Av.	June 9–12	June 21–24	July 5–8	July 19–22	Av.
Little Club.....	8.8	10.0	12.9	16.2	12.0	4.7	3.4	8.6	9.2	6.5	9.5	9.5	12.9	15.4	11.8	15.5	13.0	15.9	15.4	15.0	6.26	5.91	5.99	6.16	6.08
Marquis.....	8.6	11.0	16.1	20.3	14.0	2.0	4.0	7.0	5.6	4.6	9.6	9.6	14.7	18.1	13.0	15.8	13.4	16.3	14.5	15.0	6.24	5.92	6.20	6.32	6.17
Mindum.....	11.7	11.2	14.2	19.3	14.1	2.6	5.0	7.4	10.0	6.2	11.8	10.4	15.3	19.9	14.3	15.2	13.1	19.4	15.6	15.8	6.34	6.08	6.12	6.14	6.17
Kubanka.....	11.2	10.4	13.7	17.9	13.3	2.8	4.1	6.9	10.2	6.0	11.4	10.2	15.2	18.4	13.8	15.5	14.5	21.0	17.6	17.2	....	6.00	5.91	5.98	5.96
Kota.....	11.1	11.9	15.4	19.1	14.3	2.1	5.5	6.9	9.3	6.0	11.1	10.4	16.1	19.0	14.2	14.5	14.4	20.1	16.6	16.4	6.21	6.03	6.19	6.12	6.14
Kanred.....	11.1	11.2	11.2	11.6	11.3	2.6	2.2	6.0	5.8	4.2	11.1	10.4	12.9	13.0	11.8	15.1	15.5	19.6	19.0	17.3	6.17	6.06	5.71	5.64	5.87
Common emmer..	10.2	10.0	15.0	20.8	14.0	1.0	4.3	7.1	13.3	6.4	10.1	9.4	14.9	21.3	13.9	14.0	13.7	18.0	17.2	15.7	6.35	6.31	6.29	6.21	6.29
Khapli.....	10.0	10.8	13.7	20.3	13.7	3.6	7.0	7.2	13.5	7.8	10.2	10.4	15.1	19.0	13.7	14.5	14.7	20.5	14.9	16.1	6.13	6.04	6.10	5.94	6.05

III. CULTURE AND INJECTION EXPERIMENTS TO DEMONSTRATE INHIBITING OR ACCESSORY SUBSTANCES

Experiments were carried out to test the hyphotheses of inhibiting or accessory substances in the wheat plants as possible causes of resistance or susceptibility respectively. Modification of infection in both resistant and susceptible varieties, by the use of extracts of the leaves of varieties with opposite rust reactions, was the experimental proof it was sought to obtain. If, for example, the juice of a resistant host were administered to a susceptible one, and as a result of such treatment the susceptibility of the latter were found to be decreased, it would demonstrate, assuming adequate controls, that in the tissue-fluids of the resistant host there was present some substance inhibitory to the rust parasite.

Supplementary experiments were carried out in which certain phenolic compounds which may occur in wheat plants were used instead of plant preparations. The effect of these compounds as well as of wheat-plant extracts on rust-spore germination was also tested. There was further included in the programme a preliminary experiment in which the juice of infected seedlings was injected into healthy seedlings, to show whether the fungus excretes a substance toxic to the host.

Materials and Methods

Rust Cultures

The experiments were confined to a single physiologic form of *Puccinia graminis tritici*, namely, Form 21, obtained from the Dominion Rust Research Laboratory at Winnipeg. Stock cultures were maintained on Marquis and Little Club hosts. Inoculations were made in the usual way when the plants were about ten days old, spores being applied to the moistened leaf by a spear-pointed needle, and the plants incubated in a moist chamber for 36 to 48 hours. In the later stages of the work, this was modified to the extent of using a spore suspension rather than pure water in the atomizer with which the plants were sprayed before applying additional spores with the needle.

During the winter of 1925-26, owing to a deficiency of light in the greenhouse, it was very difficult to obtain good infection. In the next two winters conditions were much improved, an adequate system of artificial lighting having been installed. Even with this assistance, infections during the mid-winter months have been less satisfactory than at other times.

Since only one strain of wheat stem rust was being cultured in the greenhouse, special precautions to maintain its purity were not needed. To make certain that it had not become contaminated from an outside source, a sample for verification was sent annually to the Winnipeg laboratory.

Host Preparations

The varieties of wheat used were Marquis and Little Club, which are very susceptible to Form 21, and Vernal and Khapli, which are very resistant. Three kinds of preparations of the leaves of these wheat varieties have been

used in the experiments, and in addition, for reasons discussed later, certain phenolic substances. The wheat-leaf preparations are described here under their respective designations.

Press-juice. The fresh green leaves of seedling plants were ground in a meat chopper, pressed out under standard conditions, and the liquid centrifuged to remove extraneous material, all manipulations being carried out with the precautions described elsewhere (48).

Extract. The press-juice was sterilized by boiling gently under a reflux condenser for 10 minutes, decanting through a filter, and receiving the liquid in a sterile flask. The flask was then plugged with cotton wool and the liquid heated again just to boiling to ensure sterility.

Filtrate. The chlorophyll was first removed from the press-juice by passing it through a No. 12 folded filter paper. The first portion of juice underwent little change, but later the filter paper became impregnated with colloidal material and a clear amber-coloured filtrate was obtained. Filtration was slow and for that reason was carried out in a refrigerator so that the low temperature might retard enzyme action and chemical changes. The filtrate to be used in culture experiments was then sterilized by passsing through a Pasteur-Chamberland filter into a sterile flask. This operation also was carried out in the refrigerator. When prepared in this way, no bacterial or fungal growth took place over a period of 14 days in nutrient agar plates to which a few cc's of filtrate had been added. For injection experiments, the Pasteur-Chamberland filtration was found to be unnecessary and was omitted in the later stages of this part of the work.

The above three preparations have in turn been administered in three ways: (a) by adding them in various concentrations to water cultures of wheat seedlings; (b) by adding them to petri-dish cultures of wheat leaves; (c) by direct injection into the leaves of plants growing in ordinary soil.

The raw press-juice, as we had expected, proved difficult to use in culture work, on account of the rapid growth of various extraneous organisms. This difficulty was overcome in the water cultures, by changing the whole solution frequently. We considered the effort to use raw juice worth while, as one cannot be sure that the substance or system on which the rust reaction may depend is not destroyed by heating, or even by filtering.

These culture and injection experiments will now be discussed in more detail.

Water Cultures

Ward (67) used water cultures in the development of sterile seedlings for rust investigations, and Mains (42) adapted Wards' method for studying the effects of certain substances on rust development in maize seedlings. The simplicity of this method recommended its use in testing the possible absorption of effective substances through the roots.

Wide-mouthed four-ounce jars of amber glass made suitable containers. "Parowax" covers were perforated to hold eight seeds each, the seeds being held in place by cotton-wool plugs moistened to promote germination. Usually at least five healthy plants were produced in each jar. Knop's full nutrient

solution was used throughout, the various host preparations being added as desired. After press-juice had been added, it was found necessary to change the solutions very two days, or even daily in very warm weather, to prevent fermentation.

Preliminary experiments were carried out to determine what concentration of press-juice might be safely added to the cultures and at what stages of growth of the seedlings, the latter being inoculated with rust at the usual time. Concentrations of over 10 per cent strongly inhibited root-growth, and at 15 per cent the seedlings usually died at about 12 days, when the food reserves of the seed had been exhausted. At 10 per cent concentration, good seedlings were obtained if the juice was not added before the roots were an inch long, but rust infection was reduced. At concentrations of 5 per cent or less, growth was apparently normal whether the juice was first added on the day of the set-up, at germination, three days after, a week after, or nine days after, and rust pustules developed prolifically on susceptible varieties.

The toxicity of tissue juices for cells of the tissue has been reported recently by Prát (56), who found that cells of potato tubers, of the epidermis of Bryophyllum, and of onion scales can live in tap water or diluted sea water much longer than in their own juice. Nevertheless we found that a concentration of 7 per cent press-juice had no pronounced effect on wheat seedlings in water cultures, and used this in most of our experiments.

Twelve experiments were carried out, involving inoculations and observations of as many as 250 leaves in individual cases, and using both press-juice and filtrate in the culture solutions. Double controls were employed: for example, if Marquis seedlings were being tested with Khapli juice, the set-up would include cultures to which Marquis juice had been added, as well as cultures to which no juice had been added. We did not, however, succeed in getting clear-cut evidence of reduction in infection of the susceptible Marquis and Little Club varieties by the addition of preparations of the resistant Khapli and Vernal emmer varieties, or of increased infection with the conditions reversed. Consequently, the water-culture method was for the time being abandoned.

Petri Dish Cultures

Blackman and Welsford (8) made use of petri dishes in examining the infection of *Botrytis cinerea* on leaves of the host plant. They washed the leaves with sterile water and laid them on damp filter paper in the sterile dishes, inoculation being carried out at this stage. Mains included in the work already referred to (42) a study of rust infection of leaves floated on nutrient solutions in glass capsules. Clinton and McCormick (17) developed the petri dish method of rust culture, and found it satisfactory for all hosts with a hardy leaf, though less suitable for grasses. In their method the leaves were supported on glass rods or rubber bands, out of contact with the water which was placed in the bottom of the dish to maintain a humid atmosphere.

Since in our work it was desired to bring about absorption by the leaves, it was obvious that these must be in contact with the solution. Comparisons

were made of the rust infection obtained when the leaves were floated on the solution, as in Mains' method, or surpported on glass rods out of contact with water, as in the method of Clinton and McCormick, or, in a compromise between the two methods, supported on glass rods with the cut base dipping into the solution. Where contact with the substrate was maintained, we compared again four different substances: distilled water, 6 per cent sucrose solution, Knop's full nutrient solution, and nutrient agar. The last was used only where the cut base of the leaf was submerged. These experiments showed that the method of supporting the leaves on glass rods, with the cut ends dipping into 6 per cent sucrose solution, gave the best rust infection and was in other respects most satisfactory.

Two variations of the petri dish method were tried. The first was much like Ward's method of growing sterile seedlings, the leaves being placed in large test-tubes plugged with cotton-wool, with the cut bases immersed in the solution. In the second, the leaves were left attached to the stems, the latter passing through the cotton-wool plugs of test-tubes into the solution. Neither of these methods gave more than scanty infection, and bleaching of the leaves was more rapid than in the petri dish method.

The greatest difficulties to be overcome in any of these methods were the development of foreign fungi and bleaching. Various methods of sterilization were tried, but none proved as satisfactory as simply careful washing and handling of the leaves. The substrate had an important effect in this connection, sucrose being decidedly superior to the others in discouraging foreign growth. After some experience we found in fact no difficulty in culturing the rust on leaves in petri dishes, without the appearance of other organisms, when we adhered to the use of sucrose alone. The addition of plant-juice preparations, however, greatly stimulated such contaminating growth, usually dominated by *Rhizopus nigricans*. The use of raw press-juice was early abandoned because it introduced as well as stimulated contaminating organisms. Sterile filtrate, to which we then had recourse, proved much better, but even the addition of this usually stimulated some foreign growth, showing the apparent impossibility of washing the leaves completely free of adhering spores. As the cut end of the leaf was the most frequent centre of extraneous infection, the practice was adopted of selecting terminal leaves of such a size that they could be cut with a portion of the stem remaining, thus separating the cut surface as far as possible from the point of rust inoculation. Generally it was possible to keep leaves for the required two-week period of an experiment with only slight and localized foreign growth.

The effect of light intensity on bleaching was tested by placing four dishes in direct light and four under a white cloth screen in the greenhouse, and four in the laboratory where the light was less intense than for either of the greenhouse series. The rapidity of bleaching was directly proportional to light intensity. In the two greenhouse series the leaves died in four and seven days respectively, while in the laboratory they continued to live satisfactorily and rust pustules developed. No foreign fungi appeared in any of the dishes.

The substrate was found to have an important effect in this regard also. Sucrose was much superior to the others in retarding bleaching as well as in discouraging foreign growth. The possible effect of a drier atmosphere was tested by raising the covers of the dishes slightly by means of rubber bands. This, however, resulted in an increase in the number of extraneous fungi, and no reduction in bleaching was observed.

An interesting feature in connection with the bleaching of infected leaves was the persistence of islands of green tissue surrounding each pustule when all the rest was white, yellow or brown. This stimulation of host cells by the invading parasite has been referred to in the introductory portion of this paper.

Of still more interest, perhaps, was the observation that the stimulation of the growth of foreign fungi by the sterile filtrate of the wheat-plant juice was comparatively slight with filtrate of the two rust-susceptible varieties and much greater with that of the two rust-resistant varieties, being decidedly greatest in fact with the filtrate of Khapli, the most resistant variety. Why the fluids of tissues which inhibit stem-rust growth should stimulate the growth of *Rhizopus nigricans*, for example, is not at present understood. The substances concerned are apparently inactivated by boiling, since with the juice extracts little effect of this sort was observed.

In this connection reference may be made to a recent paper by Allison (4). This investigator found that extracts of twenty-six different plant materials, when used in proper concentration, all stimulated to some extent the growth of *Bacillus radicicola* on artificial media. The activity of the extracts varied widely, however, even of those from different parts of the same plants.

Method Adopted

As the method finally adopted for the petri dish culture of wheat stem rust was reached only after considerable experimentation, its presentation here in summarized form may be of use to other workers.

In preparation for an experiment the petri dishes were sterilized with the glass rods in place. A sucrose solution of 6 per cent concentration was placed in 10-cc. portions in test-tubes plugged with cotton-wool, and sterilized in an autoclave. This permitted convenient and rapid transfer to the petri dishes later. It was of course not practicable to prepare the plant juice in this way. The sterile filtrate was introduced, also in 10-cc. portions, by sterile pipettes, a separate pipette for each dish.

The terminal leaves of seedlings 2 to 3 weeks old were cut under water, with a piece of stem remaining attached whenever the size of the leaf permitted. The leaves were thoroughly washed in a gentle stream of tap water, drawing them gently through the fingers to insure the adhesion of a film of water for promoting the later germination of rust spores. They were then rinsed in a stream of sterile distilled water, and placed on a similarly washed glass plate. Using now sterile forceps and a flamed needle they were inoculated on both dorsal and ventral surfaces at a point about midway between the base and the

tip, and transferred as quickly as possible to the petri dishes. The cut end was placed between the glass rods on which the leaves rested, where it would be well covered by the nutrient solution.

When three leaves had been placed in a dish, the culture solutions were added promptly. The dish was then placed in a dark cupboard for 36 hours and then removed to the laboratory shelf. Pustules usually appeared in five to seven days and an experiment was discontinued at the end of two weeks. Four petri dishes were used for each treatment in any given experiment.

Results

No results obtained with raw press-juice are discussed here, the use of this preparation in petri dishes having been discarded on account of the difficulty with foreign fungi.

The experiments in which it was attempted to induce susceptibility to rust infection in normally resistant varieties were uniformly unsuccessful, regardless of what preparation of the susceptible hosts was employed. This suggests, so far as the results can be accepted, that susceptibility does not depend on the presence of an accessory substance in the host.

Experiments with the conditions reversed showed the boiled juice-extracts of resistant varieties to be ineffective in transferring resistance to susceptible varieties. Comparisons were made with primary controls, in which the petri dishes contained only sucrose solution, and with secondary controls, in which the dishes contained equal parts of sucrose solution and extract of the same variety to which the test leaves belonged. Rust infection of the test leaves, as judged by the development of pustules, was as great at least as that of the secondary controls.

TABLE II.

Results of adding resistant filtrate to petri dish cultures of susceptible leaves

Test variety	Resistant host prep.	Primary control	Secondary control	Test leaves
Marquis	Khapli filtr.	Excellent infection (25 pustules in 5 days)	Good infection in 5 days	1 very small pustule in 8 days
Marquis	Vernal filtr.	Excellent infection in 5 days	Good infection in 5 days	No pustules
Little Club	Khapli filtr.	Excellent infection in 5 days	Good infection in 5 days	No pustules
Little Club	Vernal filtr.	Excellent infection in 5 days	Good infection in 5 days	No pustules

The results obtained by the addition of resistant juice filtrate to cultures of susceptible leaves are summarized in Table II. These appear at first glance to leave no doubt as to the inhibiting effect of the resistant host preparation on rust development. There are, however, some objections which may be made to this conclusion.

The addition of the filtrate to culture solutions caused slightly more rapid bleaching of the leaves. This apparent toxicity, causing a reduction in the vigour of the leaves, was reflected in the slightly smaller and less numerous pustules which developed on the secondary controls. If we can assume that the filtrates of resistant and susceptible plants possessed an equal degree of toxicity to leaf tissues, and we had in fact no evidence to the contrary, this objection may be considered as satisfactorily met by the use of the secondary controls.

More difficult to dispose of is the objection arising from the obviously greater stimulation of foreign growth by the resistant filtrate. The influence of these contaminating organisms in the cultures on the rust development of the wheat leaves may not of course have been very great. The intruders were all saprophytic fungi, and even in cases where their growth was greatest it was evident that the leaves were not attacked. However, it is impossible to say definitely what may have been the effect, and until further experiments with completely sterile leaves can be carried out, this objection remains valid.

All that can be said, therefore, is that the petri dish culture experiments gave indications of the presence in resistant filtrate of a substance inhibiting rust development.

Injection Experiments

The third method used in the effort to demonstrate inhibiting or accessory substances in plant juices was that of injecting these fluids directly into leaves remaining attached to plants growing in soil.

Rumbold (60) reviewed in 1920 the literature on injections and showed that various chemicals have been successfully introduced into trees but that most workers were of the opinion that injections are either detrimental or non-effective. In an effort to control the chestnut bark disease caused by *Endothia parasitica*, she injected chestnut trees with a variety of chemical substances by boring a hole in each tree trunk and sealing a glass tube in the aperture so formed.

Lipman and Gordon (39, 40) developed a similar method of injecting trees. A glass tube connected to a raised reservoir was sealed in a hole bored in the trunk and the liquid was supplied to the conducting elements by the force of gravity. By this treatment they were able to correct the chlorosis of leaves of lemon trees and they suggested that the method could also be used to free trees from insect pests and fungal parasites, and to protect them from frost damage by injecting soluble substances capable of reducing the freezing point of plant sap.

Bews and Aitken (6) used an injection method for measuring the size of the aeration system of the leaves of plants. They injected water by placing the leaf in a tube from which they evacuated the air by means of a suction pump

By means of a needle, Reiche (58) injected cell sap, both filtered and containing plasma particles, into the intercellular regions of stems, petioles, and leaves of various species of plants, using tap water as a control. He also infiltrated

cut parts with a suction pump. The stimulation of resting cells resulted in cell division and growth, or callose proliferation; that is, he obtained a typical wound reaction. Nicolau (50) used a similar method in the injection of various substances into ten species of plants having tubular stems. Almost all the solutions he used were absorbed and circulated in the plants. Some substances, like sugars, were nutritive in their effect; others, like copper salts, were toxic. It is of interest that he found full-grown plants very many times more resistant to poisoning than seedlings.

McLean (45) in 1927 modified the porometer used by Darwin and Pertz for the study of stomatal openings so that it could be used for injecting manganese into leaves. His apparatus was very simple, consisting merely of a glass medicine-dropper with a rubber lip on the large end so that it could be pressed against a leaf without causing injury to it, and the other end was connected with a rubber atomizer bulb. When the tube was filled with dilute manganese solution and then pressed against a leaf the liquid could be pumped into the intercellular spaces through the stomata.

Methods and Materials

Although the work of neither Reiche nor Nicolau cited above had yet come to our attention, we used in our first work an ordinary glass surgical syringe, fitted with the finest-pointed hypodermic needle procurable (No. 00). The leaf to be injected was held in the left hand, stretched flat over the index finger. The hypodermic needle was then placed with the slanted edge of the needle point flat against the leaf where it was supported by the index finger. The application of gentle pressure to the plunger caused the needle to puncture the epidermis and the injected liquid to flood the leaf tissue. Successful injections were evidenced by a darkening of the leaf in the regions where the fluid penetrated. With seedlings two weeks old, three injections at different points would flood the entire leaf. The greatest care was necessary in making the injections, first, to avoid tearing the tissues unduly and, second, to prevent the rupture of large numbers of cells by too vigorous pressure on the plunger. However, some damage to the leaf was inevitable, and small dead patches formed at the points of injection. These barred the path of the liquid in later injections of the same leaves, making insertions at more points necessary, and thus causing cumulative damage. Consequently we had recourse to other methods.

Preliminary experiments with the injection method of Bews and Aitken showed this to be rather ill adapted for our purpose. Detached leaves may be placed in a convenient container for exhausting their air spaces. With whole plants growing in soil the problem is not so simple, though we grew the seedlings in glass tubes to reduce the difficulties to a minimum.

Until the appearance of McLean's paper in 1927, the porometer method published by Darwin and Pertz in 1911 had escaped our notice. Its obvious advantages for our purpose led to its immediate adoption and use in all subsequent experiments. A short piece of rubber tubing was fitted over the end of the syringe in place of the hypodermic needle, and fixed in place with a

little sealing wax. By applying pressure to the plunger, liquids could be forced to penetrate the leaf through the stomatal openings. With this instrument injections could be made simply and rapidly, and repeated any desired number of times at successive intervals without apparent mechanical injury to the leaf. During the winter, it was found that the most satisfactory time for making injections was between 1 p.m. and 3 p.m. During the summer, when light conditions were better, the stomata were sufficiently open for a longer period each day.

Experiments with plants aged two weeks, three weeks, and a month old, injected with a needle, showed that the liquid flowed most freely and evenly from the point of injection in the youngest tissue. Although this was a matter of less importance with the porometer method, the practice of using young seedlings was continued throughout. The plants were grown in four-inch pots in the greenhouse, about ten to a pot. They were inoculated at the first-blade stage, when about ten days old. In a given experiment, usually five treated pots, containing ten seedlings each, were compared with a like number of control pots, though the number was changed when necessary to suit the conditions. Where needed, secondary controls were again employed. These plants were injected either with juice of their own kind or with distilled water. Sometimes both kinds of secondary controls were used in the same experiment. The experiments were also planned to show the effect of time of injection in relation to time of inoculation, and of the frequency of injection.

The plant preparations injected were mainly raw press-juice and filtrate, the boiled extract having very soon proved to be apparently ineffective. The program of experiments with these preparations was simplified by for the most part pairing Marquis only with Khapli, and Little Club only with Vernal.

Since for reasons to be discussed in a later paper we had postulated that the effective substance, if such exists, which causes rust resistance may be a phenol, and had begun analytical studies on the phenolic compounds of wheat plants, three phenols known to occur in plants were injected in a number of different concentrations and the effect on rust development noted. The phenols used were salicylic acid, catechol and vanillin.

Results

The first experiments in which Marquis and Little Club preparations were injected into Khapli seedlings yielded no evidence of any induced reduction in the host's resistance. As this agreed with the results obtained in petri dish cultures, no further attempt was made to demonstrate the possible existence in the host of accessory substances which may be required by the rust parasite for successful infection.

The first injections of Khapli and Vernal filtrate into Marquis and Little Club seedlings, after the satisfactory perfection of the hypodermic needle technique, gave on the other hand quite encouraging indications of rust-inhibiting effects. The results of these two experiments are given in Tables III and IV. It will be observed on reference to these tables, that the pots numbered

2, 4 and 6 were secondary controls for Nos. 3, 5 and 7. Although the number of plants in the experiment was relatively small, the delay in infection and the reduction in the number and size of pustules on the leaves injected with resistant juice-filtrate seemed too regular to be merely a matter of chance. Moreover, the chlorotic area surrounding pustules, which is a characteristic feature of resistant hosts and which was entirely absent on the Little Club controls, appeared on the Little Club leaves injected with Vernal filtrate.

TABLE III.

EFFECT OF INJECTED KHAPLI FILTRATE ON RUST RESISTANCE OF MARQUIS SEEDLINGS.

Pot No.	Injected substance	Time of injection	Infection period	No. of pustules	Relative size of pustules
1	None		13 days	27 on 8 leaves	Normal
2	Marquis filtr.	2 days before inoc.	13 days	19 on 8 leaves	Normal
3	Khapli filtr.	2 days before inoc.	17 days	10 on 7 leaves	Slightly smaller than in Pot 2.
4	Marquis filtr.	On day of inoc.	13 days	18 on 5 leaves	Normal
5	Khapli filtr.	On day of inoc.	17 days	6 on 3 leaves	Small, not convergent
6	Marquis filtr.	2 days after inoc.	13 to 14 days	5 on 4 leaves	Normal
7	Khapli filtr.	2 days after inoc.	18 days	1	Very small

TABLE IV.

EFFECT OF INJECTED VERNAL FILTRATE ON RUST RESISTANCE OF LITTLE CLUB SEEDLINGS.

Pot No.	Injected substance	Time of injection	Infection period	No. of pustules	Relative size of pustules
1	None		12 days	8 on 5 leaves	Large, convergent
2	L.C. filtrate	2 days before inoc.	13 days	6 on 4 leaves	Somewhat smaller than in Pot 1.
3	Vernal filtrate	2 days before inoc.	16 days	3 on 3 leaves	Much smaller than in Pot 2.
4	L.C. filtrate	On day of inoc.	12 days	4 on 2 leaves	As in Pot 2.
5	Vernal filtrate	On day of inoc.		None	
6	L.C. filtrate	2 days after inoc.	12 days	5 on 3 leaves	As in Pots 2 and 4.
7	Vernal filtrate	2 days after inoc.	16 days	1	Minute

It can be objected, however, as in the case of the petri dish cultures, that the secondary controls also showed a reduced infection. Also it is seen that the difference between secondary controls and test leaves tends to narrow in the later injections, showing the importance of the time factor in such experiments and suggesting furthermore that the difference in the action of resistant and susceptible juice-filtrates may be one of degree rather than one of kind. There was moreover a defect in the experimental conditions which does not show in the table, namely, that the greenhouse at that time suffered an infestation of *Erysiphe graminis* which attacked the plants. Damage by this fungus was fairly uniform in all the pots, but it may have been responsible for the rather long period of infection even in the controls and perhaps also may have caused a general lessening in the severity of rust infection.

To answer these various objections, and to get information on some other points, a series of approximately 100 experiments was carried out. This enabled us to make extensive comparisons of the effects of injecting raw press-juice, juice-filtrate, phenols and distilled water. Space will not permit us to present here the voluminous tables of data from these experiments[1]. The results of a single experiment are given in Table V by way of illustration.

TABLE V.

EFFECT OF INJECTING CATECHOL FOUR DAYS AFTER INOCULATION, ON THE RUST INFECTION OF MARQUIS SEEDLINGS

Treatment	No injection					Injected with water					Injected with catechol (333 p.p.m.)				
Pot No.	(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)	(11)	(12)	(13)	(14)	(15)
No. of plants	11	10	7	7	7	10	11	10	10	8	9	8	10	9	9
No. infected	6	10	5	7	7	9	11	10	10	8	8	7	8	5	5
Per cent infection per plant.*	1	35	40	35	20	15	25	25	35	35	25	5	25	5	25
	1	35	35	25	20	15	25	25	30	35	25	5	25	5	25
	1	25	25	25	20	10	25	25	25	35	20	5	20	5	25
	1	25	25	20	20	10	20	25	25	35	15	5	20	5	20
	1	25	15	15	15	5	20	25	25	35	15	2	20	1	5
	½	20		15	15	5	20	25	25	25	15	2	20		
		20		15	15	3	20	15	15	25	10	1	10		
		15				2	15	15	10	25	5		5		
		10				1	10	5	10						
		10					10	5	5						
							10								
Av. per plant	½	22	20	21	18	7	18	19	20½	31	14	3	14½	2	11
Av. per treatment.	16.3 ± 2.7					19.1 ± 2.6					8.9 ± 1.8				

**The scale devised by the United States Department of Agriculture was used to estimate percentage infection.*

No further difficulty was encountered in keeping the test plants free of mildew. Neither was there any external evidence of toxicity of the juice or filtrate to the injected leaves; these apparently are more resistant than the

[1] *The complete tables are recorded in annual reports to the National Research Council.*

roots or than similar leaves when detached from the plant. Nothing in the nature of anaphylactic reactions was observed when successive injections were made. Probably the proteins of the two species of wheat are not sufficiently different to cause this, even if it be admitted that such reactions may be expected in plants.

The length of time elapsing between inoculation and injection had a very important effect. Injections made from two to six days after inoculation, regardless of the substance used, modified the rust infection to a greater degree than earlier or later injections. Two or more injections made at intervals of a few days were, as might be expected, more effective than single injections.

Growing conditions, especially with reference to light, influenced quite markedly the reaction of the rust to injections. When conditions were poor, and rust development rather scant, substances which reduced infection cut down both the number and size of pustules. When conditions favoured abundant infection, the reduction was less marked and manifested itself only in a lesser number of pustules. There is a close relation between vigour of host and parasite. Under flourishing conditions the parasite may be better able to withstand injected toxic substances, and furthermore the more active metabolism of the host may remove these more rapidly from the region of conflict.

A rather careful study of the effect of distilled water injections was made because of the reduction in infection of secondary controls injected with juice of their own kind: it was desired to find out, if possible, how much of this effect might be owing merely to the change in the physical or physiological condition of the plant caused by the injection of moisture. On the average, distilled water reduced infection somewhat, but under good growing conditions this was appreciable only when two or more injections were given between the second and sixth days after inoculation. No greater average reduction of infection was caused by injection of juice of the plant's own kind, but on account of the great irregularity in results, a definite conclusion on the point cannot be reached.

With the phenols, it was found necessary to use concentrations fairly close to the point at which they exhibited toxicity towards the wheat seedlings in order to cause appreciable reduction in rust infection. This varied with the phenol: with salicylic acid 200 parts per million was the limit of safety, while catechol could be used at 330 p.p.m. and vanillin at still higher concentrations.

Unfortunately this method of experiment, in spite of the various refinements introduced, is still subject to such great errors that when the results are studied statistically they do not justify definite conclusions with regard to any of the substances used. The nearest approach to definitive results was secured by the injection of phenols. With these substances, when there was an appreciable change in average rust infection it was always a reduction, even if the differences were not regular or large enough to be statistically significant.

An example of one experiment with catechol is given in Table V. The data show the average per cent infection of the primary controls (no injection) to have been 16.3 ± 2.7; of the secondary controls (injected with water), 19.1 ± 2.6; of the test plants (injected with catechol), 8.9 ± 1.8. As we are not justified by the results either of this experiment or of many others in regarding the increased average infection of the secondary controls as showing a real difference from the primary controls, we must in this case compare our test plants with the primary controls. The average reduction in per cent infection caused by the injection of catechol in this experiment is thus seen to be 7.4 ± 3.1. As this difference is less than three times the probable error, it cannot be regarded as statistically significant.

The fact, however, that we did in several cases succeed in bringing about some reduction in infection by the use of phenols and of wheat juice gives ground for hope that the way has been opened for further and more conclusive experiments. Particularly do the results obtained with phenols justify us in pursuing our analytical studies of wheat plants with reference to their content of phenolic substances. The necessity of injecting phenols in concentrations approaching the margin of toxicity to wheat leaves in order to reduce infection markedly may of course raise a doubt as to whether the effect was direct or indirect. The reduction may have been the result of diminished vigour of the host. This, however, does not necessarily affect our general hypothesis, since whatever substance is set free when the parasite penetrates a resistant host cell does in fact kill the host cell as well as the parasite.

Injection of Juice from Infected Seedlings

It will be recalled that some of Miss Allen's cytological evidence (1, 2, 3) referred to earlier made it appear probable that the wheat rust parasite excretes a toxin which is responsible for the death of the resistant host cell after penetration by the fungous haustorium. To test this point, the juice of infected Marquis seedlings was injected into healthy leaves of 10-day-old Khapli seedlings. Healthy Marquis seedlings were similarly injected as controls. The uredinia had been rubbed from the surface of the infected Marquis leaves before grinding, and the press-juice was filtered before use.

If the rust had excreted a toxin in any appreciable quantity, the injected leaves of the hypersensitive Khapli would have been expected to show some effect. The gradual adjustment sometimes observed between the parasite and a resistant host in the cells more remote from the original point of infection, and the ring of deeper green tissue surrounding pustules on the more tolerant susceptible hosts, suggest that the effect of the fungous excretion may be either stimulation or injury, depending upon the concentration of the active substance. In the present instance, however, the injections had no apparent effect whatever on the leaves of either variety. The foregoing hypothesis therefore received no support from this preliminary experiment.

It is possible, of course, that the Marquis leaves used in the preparation of the juice-filtrate were not heavily enough infected to yield an effective concentration of the fungous toxin. They showed in fact only moderate

infection, in spite of the effort to inoculate them as heavily as possible. In the process of grinding and pressing out, the fluids of the whole leaf would become intimately mixed, evidently a far different situation than obtained in the original leaf, where any toxic excretion of the parasite would be more concentrated in the immediate vicinity of each pustule. It seems worth while to make further experiments with more heavily infected leaves; also to attempt by microtechnical methods the withdrawal of fluids from cells adjoining pustules and localized injections into healthy tissues.

Proposed Cytological Studies of Injected Leaves

In the method of injection adopted for these experiments, it was necessary to assume that substances reaching the intercellular spaces were at least to some extent absorbed by the cells. Nicolau (50) and Reiche (58), to whose experiments reference has already been made, found this to be apparently the case. Nevertheless it was felt that the effects found by external observation should be checked by cytological studies. It is important to know whether in cases where reduction of infection took place the fungous haustoria actually penetrated the cells, or whether the progress of the hyphae was prematurely checked by the presence of foreign matter in the intercellular spaces. Physical effects of the injections on cells of the host, if such resulted, may also be observed in this way.

Accordingly, leaf material was embedded at various stages in several of the experiments. Up to the time of writing it has not been possible to study these preparations microscopically. A report upon them must therefore be deferred to a later paper.

Effect of Host Preparations and Phenols on the Germination of Rust Spores

In the introduction to this paper, reference was made to the experiments of Brown (14, 15) and Noble (51) on the stimulation of spore germination in *Botrytis* and *Urocystis* by substances contained in the host plants as well as by simple chemical substances. Both of these authors found also that substances which in high dilutions stimulated might in greater concentrations delay germination. Recently, O'Connor (52) has reported the inhibition of the growth of foreign pollen by substances occurring in the cells of angiosperms. For the simple, diffusible, thermostable substances responsible he suggests the name "speciamine". "Doubtless", he states, "in plants at least, the speciamines constitute, apart from mechanical protective tissues, the chief natural defence against invasion (by fungi and bacteria)."

A few preliminary experiments were carried out on the effect of wheat-plant extracts and phenols on the germination of the urediniospores of *P. graminis tritici*, Form 21. The media used were undiluted press-juice and filtrate of Khapli and Marquis, and solutions of salicylic acid, catechol and vanillin, in various concentrations. The spores were dusted on the surface of these liquids in syracuse dishes and allowed to stand at room temperature for two

days, after which time germination counts were made. Distilled water was used as a control throughout, spores on this medium giving usually about 85 percent germination.

On the press-juice of Marquis and Khapli no differences were observed in the percentage germination of spores. In both cases this was slightly reduced as compared with that of the controls. On the juice-filtrate no germination whatever was obtained with either lot. This experiment was repeated a second time with the same result. No explanation can be offered at present for this lethal effect of the filtrate towards rust spores, and it is interesting to recall the stimulation of saprophytic growth found on the addition of filtrate to petri dish cultures, an effect especially marked with resistant filtrate. These phenomena will be the subject of further investigation. Possibly dilution of the filtrate may lead to quite different results.

With the phenols, no spores germinated at concentrations greater than 50 parts per million of salicylic acid, 200 p.p.m. of catechol, and 250 p.p.m. of vanillin. This order of relative toxicity corresponds with that found for the same substances towards wheat plants, as reported on an earlier page, and towards *Helminthosporium sativum*, as reported in the next section of this paper. The possible stimulation of germination by high dilutions of these phenols has still to be investigated.

Effect of Phenols on the Growth of *Helminthosporium Sativum*

The obligate parasitism of *P. graminis* has so far made it impossible to test the effect of any substance upon its growth by culturing it on artificial media. Furthermore, as we have seen, the administration of foreign substances to the rust fungus growing in its natural medium, the wheat leaf, is beset with difficulties. These may perhaps be overcome by further experimentation. In the meantime, since we had postulated that phenols occurring in wheat plants might cause rust resistance, it was desired to obtain information as to the effect of these substances on the growth of some fungus which could be cultured more conveniently. *Helminthosporium sativum* was selected for this purpose, and experiments were made with salicylic acid, catechol and vanillin.

Cultures were grown in 200 cc. Erlenmeyer flasks containing 50 cc. of nutrient solution. In the first experiment with salicylic acid, Knop's nutrient solution was used, with the addition of 5 per cent ofsucrose. In all other experiments this mixture was replaced by Czapek's nutrient solution, which itself contains 3 per cent of sucrose. Stock solutions of the phenols were made up in a concentration of 0.1 per cent, sterilized by passing through a Pasteur-Chamberland filter, and added to the cultures in various quantities to give the desired dilutions. Duplicate flasks were used for each dilution in the first experiment, and triplicate flasks in the second experiment, with each phenol. The cultures were inoculated as usual with a platinum loop, and allowed to stand at room temperature for about seven days. They were then filtered through a Büchner funnel, and the mycelium was dried and weighed.

TABLE VI

WEIGHT OF MYCELIUM OF *Helminthosporium sativum* PRODUCED IN CULTURES CONTAINING PHENOLS

Salicylic acid				Catechol				Vanillin			
Experiment 1		Experiment 2		Experiment 1		Experiment 2		Experiment 1		Experiment 2	
Concn.	Av. wt. of dupli.	Concn.	Av. wt. of tripli.	Concn.	Av. wt. of dupli.	Concn.	Av. wt. of tripli.	Concn.	Av. wt. of dupli.	Concn.	Av. wt. tripli.
p.p.m.	mg.	p.p.m.	mg.	p.p.m.	m.g.	p.p.m.	mg.	p.p.m.	mg.	p.p.m.	mg.
0.0	42	0.0	63	0.0	45	0.0	29	0.0	64	0.0	24
1.2	57	4.0	54	13.2	45	4.0	32	1.3	72	4.0	25
11.9	74	7.9	58	19.6	76	7.9	32	13.2	83	7.9	24
19.6	70	11.9	98	32.2	80	11.9	33	26.0	115	11.9	24
38.5	90	15.4	115	62.5	52	19.6	40	62.5	114	15.4	31
90.9	No growth	19.6	121	142.0	No growth	29.2	43	117.6	100	19.6	25
		38.5	76			38.5	45			29.2	27
		56.5	No growth			47.6	50			38.5	28
		74.1	No growth			56.5	32			47.6	40
						65.4	35			56.5	33
						74.1	34			65.4	27
										74.1	26

The effect of various concentrations of the phenols on the weight of mycelium produced is shown in Table VI. In examining this table it should be borne in mind that while all the cultures in any particular experiment were grown under uniform conditions, these varied slightly in different experiments and consequently they cannot be fully compared one with another.

It is clear from the results that there is a curve of stimulation and inhibition. The first result of adding phenols in minimum effective concentration is a stimulation of growth. This increases with concentration up to an optimum and then decreases to the maximum limit of tolerance. The experiments have not been carried far enough to define this curve completely or precisely. Indeed it may be questionable whether the cardinal points can ever be defined precisely. The results presented here suggest that the optimum at least may vary with other conditions of the experiment.

The optimum concentration for growth apparently falls somewhere in the neighbourhood of 40 parts per million. However, the three phenols used vary in the intensity of their effect in the order: salicylic acid, catechol, vanillin. This is the order already noted of their relative toxicity towards wheat plants and towards the germination of rust spores. Further experiments may confirm the preliminary indication also noted, that they act in the same way towards the rust fungus growing in the wheat leaf.

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THE HYDRATION OF THE ALUMINATES OF CALCIUM [1]

I. A NEW CRYSTALLINE FORM OF HYDRATED TRICALCIUM ALUMINATE

By Thorbergur Thorvaldson[2] and Norman S. Grace[3]

Abstract

In an investigation of the resistance of Portland cement to sulphate action a new form of hydrated tricalcium aluminate, with six molecules of water, was prepared by treating pure tricalcium aluminate with steam under pressure and drying the product over quicklime. This hydrate is crystalline and isotropic. It is stable at 100 deg. C., but at 275-300 deg. C. it gives up three-quarters of its water, forming a stable 1.5-hydrate. Some water is retained up to 1000 deg. C. Before the last of the water is expelled decomposition of the aluminate takes place. Recombination occurs at 1000-1100 deg. C.

Introduction

Tricalcium aluminate is generally considered to be one of the major components of commercial Portland cement clinker (1, 2). It is of special interest in the study of the action of sulphate solutions on Portland cement and of the so-called alkali disintegration of concrete, since it has been shown (3) that mortars made of tricalcium silicate or beta dicalcium silicate and sand are stable in solutions of sodium sulphate, while the addition of tricalcium aluminate renders the mortar at least as vulnerable as Portland cement to the action of the solution. Thus, if one accepts the conclusions of Rankin (1) that properly burned Portland cement clinker is composed mainly of tricalcium silicate, beta dicalcium silicate and tricalcium aluminate, one is led to the conclusion that the destructive action of sodium sulphate on Portland cement is due to the action of the sulphate on the tricalcium aluminate in the cement.

It has also been shown (4) that proper steam curing of Portland cement sand mortars renders them immune to the disintegrating action of solutions of sodium sulphate, and that mortars so treated behave towards solutions of magnesium sulphate in the same way as mortars made of tricalcium silicate and beta dicalcium silicate without the presence of any compounds of alumina. Accepting the conclusions of Rankin as to the components of Portland cement it seems to follow that proper steam curing in some way modifies the condition of the tricalcium aluminate in Portland cement so as to make it immune to the action of sodium sulphate and so unreactive in solutions of magnesium sulphate that its stability comparesfavourably with the stability of the hydrated silicates.

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Contribution from the laboratories of the University of Saskatchewan, Saskatoon, Canada, with financial assistance from the National Research Council of Canada. The present paper is the first of a series. It represents part of a thesis presented by the junior author in partial fulfilment of the requirements for the degree of M.Sc. in chemistry, at the University of Saskatchewan.
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It is therefore of great interest to study carefully the changes which take place when tricalcium aluminate is hydrated at ordinary temperatures, and when it is treated with steam under conditions which render ordinary Portland cement mortars immune to the action of solutions of sodium sulphate.

Several formulas have been suggested for hydrated tricalcium aluminate obtained by hydration in water at ordinary temperatures. Gallo (5) concludes that the compound $3CaO.Al_2O_3.10H_2O$ is present in pozzolana mortars. Klein and Phillips (6) report the composition $3CaO.Al_2O_3.5\frac{1}{2}H_2O$ for the hydrate dried at 100 deg. C., the formula $3CaO.Al_2O_3.9H_2O$ for the hydrate dried over sulphuric acid and $3CaO.Al_2O_3.10\frac{1}{2}H_2O$ for the hydrate formed in a solution of calcium hydroxide with subsequent drying over sulphuric acid. Pulfrich and Linck (7) state that they obtained a hydrate of the formula $3CaO.Al_2O_3.7H_2O$. The lack of agreement between the results of these experimenters suggests that further study of the hydration products of tricalcium aluminate is necessary.

Preparation of Materials

Calcium Carbonate

C.p. calcium carbonate was dissolved in pure nitric acid. The neutral solution was treated with bromine water and milk of lime in excess, filtered, and the calcium nitrate recrystallized several times, a centrifuge being used to separate the crystals from the mother liquor. The calcium was then precipitated as the carbonate from a dilute solution of the nitrate using redistilled ammonium carbonate, washed thoroughly by decantation, filtered, washed and dried in a platinum dish.

Aluminum Hydroxide

Aluminum hydroxide was precipitated from a boiling two-per-cent solution of ammonia alum which was free from iron, using redistilled ammonia water; the precipitate was washed thoroughly by decantation, filtered off, washed, redissolved in pure hydrochloric acid, the solution diluted to the original volume and the precipitation as aluminum hydroxide repeated. After thorough washing, the aluminum hydroxide was dried in a covered platinum dish and ground to pass a 200-mesh sieve. The material had an ignition loss of about 30 per cent.

Tricalcium Aluminate

Sample 1. Calcium carbonate and aluminum hydroxide, prepared as described above, were mixed wet in the proportions $3CaO:Al_2O_3$. The water was then evaporated with constant stirring of the mixture to prevent segregation. When dry, the material was transferred to platinum crucibles, placed in a platinum-wound resistance furnace, the temperature gradually raised to 1300 deg. C. and kept there for several hours. The sintered mass was then broken up, ground in an agate mortar to pass a 200-mesh sieve, and the heating repeated, the temperature being raised to about 1370 deg. C. The material was repeatedly ground and reheated until White's test (8) indicated absence

of free lime, and careful examination by the petrographical microscope showed that the substance was homogeneous and free from particles of lime and $5CaO.3Al_2O_3$.

Microscopic examination showed that even after several heat treatments at 1370 deg. C. the material contained small amounts of lime and $5CaO.3Al_2O_3$. It was found that treating the finely powdered material with water aided combination materially during the subsequent heat treatment, due probably to the activation of the free lime. The treatment, however, seemed to cause tiny voids to be formed in the crystals of tricalcium aluminate and this made exact microscopic examination more difficult. These voids were eliminated by subsequent heat treatments.

The final product was free from CaO or $5CaO.3Al_2O_3$. It was composed of homogeneous, isotropic crystals, with a refractive index of 1.710 ± 0.002.

Sample 1G. This sample was prepared from a good grade of calcium carbonate and alumina. The method of preparation was similar except that the material was fired in the form of a hollow cylinder standing on a magnesia disc in an oil-fired furnace. The material in immediate contact with the magnesia disc was discarded after each firing. This sample had a slight excess of alumina, and contained, according to microscopic examination, a small amount of $5CaO.3Al_2O_3$ but no free lime.

The samples were kept in carefully sealed bottles to prevent absorption of moisture. Chemical analysis of these samples gave the following results:

TABLE I.*

CHEMICAL COMPOSITION OF PREPARED SAMPLES

Sample No.	1	1G	Theoretical $3CaO.Al_2O_3$
CaO	62.31	60.89	62.27
Al_2O_3	37.76	39.05	37.73
Fe_2O_3	Absent	Absent	
MgO	Absent	Trace	
SiO_2	Absent	0.06	

* *Analyses by David Wolochow.*

Retention of Water by Hydrated Tricalcium Aluminate on Ignition

In the preliminary experiments on the hydration of tricalcium aluminate the observation was made that the value for the percentage of the water of hydration, calculated from the gain in weight of the original anhydrous material during hydration, was always higher than the loss on ignition over the blast lamp or in an electric muffle at 800-900 deg. C.

Determinations, by means of a Leeds and Northrup optical pyrometer, of the temperature to which the platinum crucibles were heated over the blast lamp, gave values varying from 1025 deg. C. to 1075 deg. C. for the bottom of

the crucible. While the upper portion of the crucible was at a somewhat lower temperature, it is probable that at least a portion of the sample of aluminate in the crucible was heated to 1000 deg. C. or more.

It was found that continued ignition, at the highest temperature obtainable over the blast lamp, expelled the remaining water gradually. A typical experiment illustrating the great difficulty of eliminating all the water of hydration follows.

Weight of anhydrous tricalcium aluminate	0.9350 grams.
Weight of crucible + anhydrous aluminate	24.5079 grams.
Weight of crucible + hydrated aluminate	24.8816 grams.
Weight of crucible + aluminate after extended heating over the blast lamp	24.5160 grams.

This corresponds to a retention of 0.87 per cent of water on the basis of the weight of anhydrous aluminate. The weights after successive ignitions, at first for 35-minute periods, later for 1½-hour periods were: 24.5156, 24.5153, 24.5144, 24.5134, 24.5132, 24.5130, 24.5124, 24.5121, 24.5117, 24.5113, 24.5112, 24.5112. The last weight corresponds to a loss of 0.35 per cent less water than the amount which had been taken up during hydration. The crucible was then heated for two hours at 1300 deg. C. in a platinum-wound resistance furnace. The weight was then 24.5074 grams, which is 0.5 mg. less than the initial weight of the crucible and anhydrous aluminate. Assuming that all the water of hydration was expelled, there was a loss of 0.5 mg. of platinum. As this sample was rehydrated and heated a number of times before it was removed from the crucible the loss of platinum could not be checked.[1]

As it was of the utmost importance in this investigation to be able to determine, without appreciable error, the water of hydration of a sample of hydrated tricalcium aluminate, by ignition of the material, a further extensive series of experiments was carried out to determine the lowest temperature at which the water of hydration is completely expelled. An electric furnace, the temperature of which could be fairly well controlled up to 950 deg. C., and the platinum-resistance furnace which could be adjusted within a few degrees, at temperatures above 1000 deg. C., were used. The tricalcium aluminate was hydrated quantitatively in the steam autoclave at 150 deg. C., or in water at room temperature, and then brought to constant weight over lime. The quantities of anhydrous tricalcium aluminate used in each experiment varied from 0.4 to 1.0 gram. A summary of the results appears in Table II.

[1] *The exact determination of the loss in weight of a platinum crucible during heating at high temperatures presents serious difficulty when the crucible has to be cleaned with acid, as in the above case, before weighing. Thus Burgess and Sale* (9), *determining the loss in weight of empty platinum crucibles on heating at 1200 deg. C. and washing with hydrochloric acid, found that the washing was responsible for from 5 to 68 per cent of the total loss.*

TABLE II.

RELATION OF TEMPERATURE AND DURATION OF HEATING TO WATER RETAINED BY HYDRATED TRICALCIUM ALUMINATE

Method of heating hydrated aluminate	Time of heating, hours	Number of experiments	Average moisture retained, per cent
Muffle at 700 deg. C.	16	1	1.6
Muffle at 750 deg. C.	12	1	1.4
Muffle at 800 deg. C.	4	1	1.6
Muffle at 850 deg. C.	12	1	1.2
Muffle at 900 deg. C.	5 to 15	4	1.0
Muffle at 950 deg. C.	2	1	1.1
Blast lamp	1	4	1.0
Blast lamp	>10	1	0.4
Furnace at 1000 deg. C.	2*	4	0.32
Furnace at 1100 deg. C.	2*	4	none

* *The total time during which the material was exposed to temperatures between 900 deg. C. and the maximum was much more than 2 hours, as the furnace temperature fell very slowly after the electric current was cut off.*

The results at the highest temperature were complicated by the fact that, in some cases at least, an appreciable quantity of platinum was lost. The results indicate that all the water in hydrated tricalcium aluminate is probably expelled by heating 2 hours at 1100 deg. C. in a platinum furnace (total time in furnace above 800 deg. C. about 4 hours), but that at temperatures of 1000 deg. C. and lower some of the water may be retained.

Hydration of Tricalcium Aluminate in Saturated Steam at 150 deg. C.

Freshly ignited samples of tricalcium aluminate contained in covered platinum crucibles, protected from condensing water, were placed in a steam autoclave containing water free from carbon dioxide and heated at 150 deg. C.[1] (pressure of steam approximately 70 pounds per square inch). After a period of time in the autoclave the crucibles were placed in a vacuum desiccator over quicklime until constant in weight. The steam treatment of the material and subsequent drying over quicklime were repeated until no further gain in weight was produced. It was found that while tricalcium aluminate hydrates with considerable evolution of heat when water is added, only partial hydration takes place at once, and that prolonged exposure to steam is necessary before the hydration is complete. This is illustrated by the results given in Table III.

[1] *This temperature was chosen because it has been found in this laboratory that Portland cement mortars thus treated become very rapidly immune to the action of sodium sulphate.*

TABLE III.

RATE OF HYDRATION OF TRICALCIUM ALUMINATE

Time in autoclave at 150 deg. C.	Water retained after exposure over CaO, as per cent of anhydrous aluminate	
Hours	*Sample 1*	*Sample 1G*
4	35.0	.....
17	39.93	.....
22	.....	35
51	39.97	.....
70	.....	39.6
94	40.14	39.98
112	.....	40.02

The purer sample, No. 1, hydrates much more rapidly than does sample No. 1G, which, however, contained only very small amounts of impurities, mainly in the form of $5CaO.3Al_2O_3$.

There was no difference in the final amount of water taken up when the material was hydrated directly in steam and when water was added to the material with subsequent steam treatment. Table IV gives the results of hydration experiments in the steam autoclave at 150 deg. C.

TABLE IV

HYDRATION OF TRICALCIUM ALUMINATE IN SATURATED STEAM AT 150 DEG. C.

Exp. No.	Sample No.	Initial weight of anhydrous sample	Weight of hydrated sample over lime	Water taken up, per cent of initial weight	Molar ratio H_2O / $3CaO.Al_2O_3$	Time in autoclave at 150 deg. C.
		grams	*grams*	*per cent*		*Hours*
1	1G	0.8673	1.2147	40.06	6.00	112
2	1G	0.9449	1.3227	39.98	6.00	94
3	1G	0.4814	0.6738	39.97	5.99	83
4	1G	0.5098	0.7148	40.20	6.03	108
5	1	0.5194	0.7279	40.14	6.02	93
			0.7281	40.18	6.03	145
			0.7286	40.28	6.04	72
6	1	0.4701	0.6587	40.12	6.02	93
			0.6588	40.14	6.02	145
			0.6589	40.16	6.02	72
7	1	0.5103	0.7148	40.07	6.01	168
			0.7149	40.09	6.01	72
			0.7150	40.11	6.01	96
8	1	0.4931	0.6903	39.99	6.00	120
			0.6904	40.01	6.00	168
			0.6915	40.23	6.03	96
Average				40.11	6.01	

NOTE.—*The second and third results given under Experiments Nos. 5, 6, 7 and 8 were obtained by ignition of the hydrated samples over the blast lamp followed by rehydration in the usual way.*

Four similar experiments were made with two other samples of tricalcium aluminate, which were equal to sample No. 1 in purity. The average result was almost identical with the one above (molar ratio, 6.02).

The dried hydrated tricalcium aluminate is hygroscopic and has therefore a marked tendency to take up moisture during weighing. The removal of the last trace of free moisture even in a vacuum over quicklime proceeds very slowly, and this adsorbed moisture may not be completely removed at room temperature. There is also the possibility of inclusions of water in the crystals of the hydrate. All these errors would tend to make the value for the water of hydration high.

The question presents itself, whether a higher hydrate may not be formed in saturated steam at 150 deg. C. and decomposed on exposure over lime in a vacuum.

Samples of tricalcium aluminate No. 1, which had been hydrated in platinum crucibles by steam in an autoclave at 150 deg. C. for 66 hours, were kept at 21 deg. C. in a vacuum desiccator over a solution of lead nitrate containing an excess of the salt. The humidity in the desiccator was therefore constant at about 98 per cent saturation (vapour tension at 21 deg. C., 18.1 mm.). Other samples were exposed similarly over a saturated solution of potassium chloride which gives a humidity of 87 per cent (vapour tension 16.0 mm. at 21 deg. C.). In all cases the amount of water contained in the material after a few days' exposure was only slightly greater than that corresponding to the formula of the hexahydrate, an excess of only two per cent being retained over a saturated solution of lead nitrate, and less than one per cent over a saturated solution of potassium chloride.

These results, together with those given in Table IV, indicate that when tricalcium aluminate is hydrated in saturated steam at 150 deg. C., or when it is hydrated in water and subsequently exposed to saturated steam at 150 deg. C., a compound of the formula $3CaO.Al_2O_3.6H_2O$ is formed. If a hydrate containing a greater amount of water were formed under these conditions, then it would have to possess at 21 deg. C., a vapour tension greater than 98 per cent of the vapour tension of water at that temperature (18.1 mm. at 21 deg. C.). The results indicate further that ignition of the hydrate $3CaO.Al_2O_3.6H_2O$, followed by rehydration of the residue in steam, gives the same product.

Hydration of Tricalcium Aluminate in Water at Room Temperature

A freshly ignited sample of tricalcium aluminate was placed in a platinum dish; distilled water, free from carbon dioxide, was added, and the covered dish was kept in a vacuum desiccator over water to prevent absorption of carbon dioxide. While partial hydration takes place very rapidly with evolution of heat, it is difficult to be certain that the last trace of the original material is hydrated, on account of the formation of a hydrated layer on the outside of the particles, preventing the access of water to the inside. The lumps were therefore broken up and the material mixed thoroughly from time to time. In 15 days, when it was estimated that hydration must be complete, the dish

was transferred to another vacuum desiccator containing freshly ignited lime, where it was kept until its weight became constant. Water was then again added and the material was allowed to remain in contact with water with frequent stirring for another period of 9 days, after which it was again dried to constant weight over lime. There was no increase in weight during the second period. The percentage of water taken up was then determined from the loss on ignition. Other samples of tricalcium aluminate were hydrated in a similar manner.

The dried hydrate was not as granular as that formed in the steam autoclave, and it was more hygroscopic. Microscopic examination showed that the particle size of the steam-hydrated material was somewhat larger. The method of preparing the water-hydrated samples made it possible that a very small amount of carbon dioxide was absorbed. Table V gives the water of hydration as determined by heating at 1100 deg. C.

TABLE V.

HYDRATION OF TRICALCIUM ALUMINATE IN WATER AT ROOM TEMPERATURE

Experiment No.	Sample No.	Weight of hydrated sample	Weight of anhydrous sample	Water determined by loss at 1100 deg. C.	Molar ratio $\frac{H_2O}{3CaO.Al_2O_3}$
		grams	*grams*	*per cent*	
1	1	0.6069	0.4341	40.43	6.06
2	1	0.5971	0.4255	40.33	6.05
3	1	0.7130	0.5090	40.08	6.01
Average					6.04

The product obtained when tricalcium aluminate is hydrated in water at room temperature, with subsequent exposure over lime till constant weight is attained, is therefore of the same composition as the hydrate obtained in steam at 150 deg. C., and may be represented by the formula $3CaO.Al_2O_3.6H_2O$. Addition of water to this product and exposure at 21 deg. C. to an atmosphere of 18.1 mm. water vapour (98 per cent saturation) did not change the composition materially.

Hydrolysis as a Possible Explanation

It might be suggested that hydrolysis of the tricalcium aluminate according to the following equation:

$$3CaO.Al_2O_3 + 6H_2O \longrightarrow 3Ca(OH)_2 + 2Al(OH)_3$$

would account for the water taken up during hydration. A large number of samples of the hydration product were tested for free lime, but invariably with negative results. The methods used were those of White (9) and of Emly as modified by Lerch and Bogue (10).[1] Furthermore it was found that aluminum

[1] *While this method has not been found to give very reliable quantitative results when used in this laboratory to determine free lime in Portland cement, it seems to give fairly consistent results when used to determine free lime in the calcium aluminates.*

oxide, which had been hydrated in the steam autoclave at 150 deg. C. and then exposed over quicklime in the same way as the hydration product of tricalcium aluminate, until reaching constant weight, attained a composition represented approximately by $Al_2O_3.H_2O$, instead of $Al_2O_3.3H_2O$. The optical examinations and the X-ray diffraction spectrum of the hydrated material to be described in a later communication, also support the conclusion that a true crystalline hydrate of tricalcium aluminate was formed.

The Stability of the Hydrate $3CaO.Al_2O_3.6H_2O$

The fact that this hydrate is stable on continued exposure in a vacuum over quicklime at room temperature, indicates that its vapour tension must be very low. A series of experiments was made in which one-half gram samples of the hydrate were heated in a platinum crucible in a vertical tube furnace through which a current of air was passed. The air was purified and dried before entering the furnace by passing it through a long column of beads wetted with a saturated solution of potassium hydroxide, then through a wash bottle containing concentrated sulphuric acid, and finally through a large tower filled with lumps of quicklime.

On heating one hour under these conditions at 100 deg. C. the sample of hydrate lost less than 0.1 per cent of its water of hydration. Further heating for three hours at 200 deg. C. expelled less than one per cent, while on one hour's heating at 300 deg. C., 75 per cent of the total water of hydration was lost. Further heating at 300 deg. C. expelled more of the water of hydration extremely slowly. When a sample of the original hydrate was heated under the above conditions at about 275 deg. C. the water was evolved at first fairly rapidly until the composition of the residue approached the value $3CaO.Al_2O_3.1.5H_2O$, when evolution of water almost ceased, a much higher temperature being required to expel the remainder of the water present. This indicates that a definite hydrate of this composition exists.

Tests for free lime were made on the residues as the dehydration proceeded. Both White's test and Emley's test were negative when the composition of the residue was represented by the formula $3CaO.Al_2O_3.1.5H_2O$, but the latter test indicated that a trace of free lime was present when the residue reached the composition $3CaO.Al_2O_3.H_2O$. After this, as the dehydration proceeded, the amount of free lime increased until a maximum of 26 to 27 per cent of CaO was reached.

The amount of free lime found in the ignited residue depends on the temperature at which the hydrated tricalcium aluminate is heated, and on the duration of the ignition. Table VI contains experimental results illustrating this.

TABLE VI.

LIBERATION OF LIME ON IGNITION OF HYDRATED TRICALCIUM ALUMINATE

Experiment No.	Method of hydration	Temperature of ignition	Time of ignition	Water retained	Free lime, per cent on basis of ignited residue
		deg. C.	*hours*	*per cent*	
1	water	700	16	1.6	26.3
2	steam	750	12	1.4	26.4
3	water	800	4	1.65	26.6
4	steam	850	12	1.2	26.2
5	steam	950	2	1.1	25.4
6	steam	Blast lamp	..	1.1	25.8
7	steam	Blast lamp	..	1.1	26.2
8	steam	Blast lamp	..	1.0	22.1
9	steam	Blast lamp	..	0.9	20.7
10	steam	1000	2	0.65	12.1
11	steam	Blast lamp	13	0.56	6.5
12	steam	1000	2	0.03	none
13	steam	1000	2	0.06	none
14	steam	1100	2	none	none
15	steam	1100	2	none	none
16	water	1100	2	none	none
17	water	1100	2	none	none

If hydrated tricalcium aluminate decomposes on ignition into the aluminate next lower in line, namely $5CaO.3Al_2O_3$ and free lime according to the equation

$$3(3CaO.Al_2O_3.6H_2O) \longrightarrow 5CaO.3Al_2O_3 + 4CaO + 18H_2O$$

then 27.67 per cent of lime, calculated on the basis of the anhydrous residue, should be liberated. While the highest quantity of free lime found was only 26.6 per cent, that is, one per cent lower than the theoretical quantity, this may easily be due to either the presence of some undecomposed hexahydrate (some residual water of hydration always remained in the samples containing free lime) or to a slight local recombination of lime with the $5CaO.3Al_2O_3$, since this takes place slowly when heated at the temperature of the blast lamp and rapidly when heated at 1100 deg. C.

When a sample of the hexahydrate of tricalcium aluminate, which has been heated until the liberation of lime is practically complete, is treated with saturated steam at 150 deg.C. in an autoclave, the free lime disappears gradually and the hexahydrate is again completely formed. The reaction can be reversed at will by ignition and steam treatment.

An X-ray diffraction spectrum of an ignited sample of the hexahydrate of tricalcium aluminate,which, according to duplicate determinations by Emley's method,contained 25.4 per cent of free lime, was found to give a pattern containing the characteristic lines of both $5CaO.3Al_2O_3$ and of CaO. On the other hand, a similar sample which had been heated at about 1000 deg. C. until nearly all the free lime had disappeared, gave the pattern of anhydrous

tricalcium aluminate as well as some of the strongest lines for CaO and $5CaO.3Al_2O_3$. (See Table VII). There seems therefore to be no doubt that the reaction which takes place when the hexahydrate is ignited is represented by the equation given above, while recombination takes place on prolonged heating at or above 1000 deg. C.

X-ray Diffraction Spectra

The photographs of the X-ray diffraction spectra were obtained by the method of Debye and Hull with a multiple-power spectrograph manufactured by the General Electric Company. A Coolidge tube with a water-cooled molybdenum target was used. The accuracy of the readings was checked by means of patterns obtained with pure sodium chloride. The photographs are not shown, but the planar spacings and relative intensity of the lines in the X-ray photographs are given in Table VII.

TABLE VII.

X-RAY DIFFRACTION PATTERNS

CaO		$5CaO.3Al_2O_3$		Ignited $3CaO.Al_2O_3.6H_2O$				$3CaO.Al_2O_3$	
				Sample 1**		Sample 2***			
Planar spacing	Relative intensity	Planar spacing	Relative intensity	Planar spacing	Relative intensity	Planar spacing	Relative intensity	Planar spacing	Relative intensity
d_{hkl}, A°		d_{hkl}, A°		d_{hkl}, A°		d_{hkl}, A°		d_{hkl}, A°	
		3.20	M	3.20	M	3.31	WW	4.10	W
		3.00	M	3.00	M	3.03	WW	3.30	WW
2.77	M*			2.77	M	2.70	SS	3.03	WW
		2.68	SS	2.67	SS	2.42	M	2.70	SS
2.40	SS	2.44	S	2.42	SS	2.20	M	2.41	W
		2.18	S	2.18	M	2.03	WW	2.20	M
		1.947	M	1.94	M	1.904	S	2.03	WW
1.69	SS	1.725	W	1.70	S	1.830	WW	1.905	S
		1.660	M	1.655	M	1.735	WW	1.830	WW
		1.600	M	1.597	M	1.700	W	1.730	WW
		1.516	W	1.516	W	1.598	WW		
1.445	M			1.450	W	1.555	S	1.555	S
1.383	M	1.390	M	1.388	S	1.450	WW	1.445	WW
		1.337	WW	1.337	WW	1.384	WW		
		1.305	W	1.307	W	1.346	M	1.347	M
		1.262	WW	1.255	WW	1.230	W	1.232	W
1.200	W	1.207	W	1.202	M	1.203	M	1.203	M
		1.172	W	1.172	W	1.101	W	1.100	W
		1.140	W	1.137	W	1.075	WW		
1.100	W	1.111	W	1.107	M	1.018	M	1.020	M
1.071	M	1.087	WW						
		1.064	WW	1.073	M	0.898	W	0.900	W
0.980	M			0.982	M	0.852	W	0.852	W
0.922	W	0.928	W	0.928	W	0.812	W	0.813	W
0.849	W			0.852	W	0.800	WW		
0.811	W			0.805	W	0.779	WW	0.778	WW
0.800	W			0.799	W	0.750	W	0.748	W
0.758	W			0.762	W	0.725	WW		
0.722	W			0.724	W	0.695	WW	0.697	WW
0.642	W			0.644	W				

* SS—*Very strong;* S—*Strong;* M—*Medium;* W—*Weak;* WW—*Very weak.*

** *Sample No. 1 was ignited at 950 deg. C. for 2 hours. It still retained 1.1 per cent of water and a determination by Emley's method (10) indicated that it contained 25.4 per cent of free lime.*

*** *Sample No. 2 was ignited over the blast lamp for an extended period. The determination for lime gave about 3 per cent CaO.*

The patterns obtained from CaO, $5CaO.3Al_2O_3$ and $3CaO.Al_2O_3$ agree very closely with the results published by Harrington (11).

From an inspection of Table VII it is evident that the product obtained by igniting tricalcium aluminate for two hours at 950 deg. C. (Sample No. 1) gave the characteristic lines for the X-ray spectrum of both CaO and $5CaO.3Al_2O_3$, and that after prolonged heating over the blast lamp (Sample No. 2) the lines for anhydrous tricalcium aluminate appeared. In the photograph of the latter sample the characteristic lines of tricalcium aluminate show up strongly, while several lines of calcium oxide show up weakly (d_{hkl}, 1.700, 1.384, 1.075, 0.800 and 0.725 A°) and only one line due to $5CaO.Al_2O_3$ (d_{hkl}, 1.598 A°) was visible as a very faint line. It should, however, be noted that several of the stronger lines for the last substance have nearly the same wave lengths as strong lines in the spectrum of tricalcium aluminate, and that changes observed in the relative intensity of some of the lines of Sample No. 2 as compared with the spectrum of pure tricalcium aluminate (especially d_{hkl} 2.42 A°) show the effect of the presence of the substance ($5CaO.3Al_2O_3$ in the mixture. The X-ray patterns thus confirm the conclusions arrived at from consideration of the results in Table VI.

Other Hydrates of Tricalcium Aluminate

Klein and Phillips (7) and others have described a crystalline hydrate of tricalcium aluminate forming hexagonal plates, needles and spherulites which are optically identical. These are described by Klein and Phillips as positive, exhibiting medium double refraction and having refractive indices, $N_e = 1.552 \pm 0.003$ and $N_o = 1.535 \pm 0.003$. The hexahydrate of tricalcium aluminate described in this paper was found to be isotropic and to have a refractive index [1], $N_{Na} = 1.604 \pm 0.002$.

A further study of these two hydrates will be reported in a later paper of this series.

Acknowledgments

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[1] *Measurements by V. A. Vigfusson.*

VACCINATION AGAINST TUBERCULOSIS WITH BACILLUS CALMETTE-GUERIN[1]

By A. C. Rankin[2]

Abstract

This paper covers work on B.C.G. vaccine carried on in Alberta over a period of approximately four years, during which time some 250 calves were made the subject of experiments. The vaccine was found to be entirely harmless, vaccinated calves developing normally and showing at autopsy no tuberculous lesions. Vaccinated animals in most cases reacted subsequently to the tuberculin test. Calves vaccinated with B.C.G. and immediately exposed to infection showed moderately increased resistance to tuberculosis over unvaccinated controls. Calves vaccinated with B.C.G. and subsequently kept under sanitary conditions for a period, in order to permit resistance to develop before exposure to infection, showed 80 per cent immunity as compared with 14 per cent for the controls. The tuberculous lesions found in vaccinated calves were in general much less pronounced as well as less numerous than those in unvaccinated animals. (F.E.L.)

Introduction

In considering any method for the control of tuberculosis in bovines, certain fundamental points should be borne in mind.

Admitting the present method of control—based on the tuberculin reaction—to be the only well tried method, one should nevertheless not overlook the fact that the method is one which takes no cognizance of racial or individual degree of resistance to the disease.

In the human we have the contrast of the disease, as it is seen in civilized man from congested communities, where almost all adults react to tuberculin, and where, in spite of this enormous extent of infection, less than 10 per cent die of the disease; compared with primitive peoples, on the other hand, where the disease when introduced is violent and a rapid fatal termination is common.

Since the observations of Marfan, in 1886, it has been recognized that protection against tuberculosis is afforded by the presence within the body of a mild tuberculous lesion which has become latent. This immunizing effect of a latent infection is well established clinically in man, and there has been adequate demonstration of the effect in cattle.

On a biological basis it is probably correct to assume that cattle which have acquired some degree of immunity, through tuberculous infection, cannot but eventually have this acquired resistance lowered in succeeding generations, in the absence of further tuberculosis infection, and, indeed,

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Contribution from the laboratories of the University of Alberta, Edmonton, Alberta, with financial assistance from the National Research Council of Canada. These studies form part of a co-operative attack on the problem of tuberculosis, carried on jointly by the National Research Council, the Dominion Department of Agriculture, Fort Qu'Appelle Sanatorium, McGill University, Queen Alexandra Sanatorium, Queen's University, the Sun Life Assurance Company of Canada, the University of Alberta, the University of Montreal and the University of Toronto.

[2] *Dean of the Faculty of Medicine and Professor of Bacteriology and Hygiene at the University of Alberta, and Chairman of an Alberta Committee on Tuberculosis of which Drs. Baker, Bow, McCullough, Ower, Sackville, Shaw, Talbot and Vango are members.*

we have indications that such is the case. The problem of infection in such non-resistant cattle can therefore be compared with that of primitive peoples, where freedom from tuberculosis is dependent on freedom from sources of infection and not on resistance to infection. When, in such a case, there is subsequently an exposure to infection, tuberculosis must result in a large proportion of the animals exposed.

If cattle, by virtue of a naturally acquired infection held in check by becoming latent, exhibit some degree of resistance to the disease, is it not reasonable to assume, theoretically, at least, that it should be feasible to definitely cause a controlled infection and accomplish the same result? There is nothing new about this, of course, and, in fact, it is easy to produce a degree of resistance in cattle.

During the period 1900-1910 many cattle were vaccinated with living tubercle bacilli of the human type, which is of low virulence for cattle. Hopes were high that such vaccination constituted the solution of the control of the disease in bovines. It was subsequently shown, however, that the resistance so produced in cattle was transient, and it was further considered a dangerous proceeding in that lesions of the udder sometimes resulted whereby virulent bacilli were present in the milk. For these reasons, as is well known to all those interested in the subject, the procedure was finally abandoned.

When the Alberta Committee on Tuberculosis took up the question of B.C.G. vaccine, in September, 1924, we did so for the purpose of investigating the claims made for the vaccine by Calmette and Guérin in their work published in the Annals of the Pasteur Institute, March, 1924, and in previous and subsequent articles. The objects of our investigations have been simply to try and determine whether B.C.G. is harmless or otherwise to bovines, and whether it is possible by this vaccine to produce resistance in healthy animals to subsequent infection.

After some preliminary preparation—lasting about six months, during which a culture of B.C.G. was obtained from Calmette and developed—the experimental part of the investigation was finally started on March 27, 1925, when the first calf was vaccinated.

Calmette and Guérin worked first with animals from 7 to 8 months old, but subsequently articles and references indicated that new-born calves might be used for the investigation. We, therefore, decided to use new-born calves in our investigations, and possibly others. We had at our disposal several stable herds and some range cattle. One of the stable herds contained under one roof 60 animals reacting to tuberculin, and we decided to use this herd for part of our investigations, in order that we might place vaccinated animals and controls in contact with tuberculous animals, and might feed them on the milk of cows which had been shown by the examination of the milk to be excreting *B. tuberculosis*.

The Vaccine

The vaccine has never been used more than 10 days old. It has been made from cultures on glycerined potato, grown at 38 deg. C. for not more than 28 days, according to the methods employed and recommended by Calmette. The dose has always been 60 mg. moist weight, injected subcutaneously into the dewlap. The swelling caused by the injection has never been painful or sensitive as far as could be determined, but has shown some tendency to vary in size. An animal injected on the 27th of March had still an appreciable lump in the dewlap in October, while one injected on the 18th of April did not show a swelling at that time. There had not been any suppuration following the injection, with one exception, and in this we found a staphylococcus in the discharge.

Tuberculin Reaction

In our first communication to the National Research Council of Canada, October, 1925, we reported that animals free from tuberculosis and not reacting to tuberculin would, when vaccinated with 60 mg. B.C.G., subsequently react with tuberculin (Table I).

TABLE I.

TUBERCULIN REACTION OF CALVES PREVIOUSLY VACCINATED WITH CALMETTE VACCINE

Date vaccinated	Tag No.	Sex	Date of testing	Tuberculin reaction	Evidence of vaccination remaining
Mar. 27, 1925	368	F	Aug. 18, 1925	Positive	Well marked pigeon egg.
Apr. 5, 1925	371	F	Aug. 18, 1925	Doubtful	No swelling.
Apr. 18, 1925	373	F	Aug. 18, 1925	Positive	No swelling.
Apr. 18, 1925	374	F	Aug. 18, 1925	Positive	Pigeon egg.
Apr. 21, 1925	377	F	Aug. 18, 1925	Positive	Pigeon egg.
Apr. 28, 1925	379	F	Aug. 18, 1925	Positive	Small swelling.
Apr. 28, 1925	381	F	Aug. 18, 1925	Positive	Pigeon egg.
May 29, 1925	382	M	Aug. 18, 1925	Positive	Pigeon egg.
May 29, 1925	383	M	Aug. 18, 1925	Positive	Pigeon egg.
May 29, 1925	384	F	Aug. 18, 1925	Positive	Pigeon egg.
June 4, 1925	385	F	Aug. 18, 1925	Positive	Pigeon egg.
May 17, 1925	303	not stated	Sept. 17, 1925	Positive	Not stated.
May 26, 1925	310	not stated	Sept. 17, 1925	Positive	Not stated.
Apr. 11, 1925	312	not stated	Sept. 17, 1925	Positive	Not stated.
May 20, 1925	314	not stated	Sept. 17, 1925	Positive	Not stated.
June 22, 1925	328	not stated	Sept. 17, 1925	Positive	Not stated.

Temperature and Development

We also reported that an injection of 60 mg. of B.C.G. vaccine into the dewlap in new-born calves was not followed by an appreciable rise in temperature, nor with any interference with normal development. Table II gives the details.

TABLE II.

EFFECT OF VACCINATING CALVES WITH B.C.G.

Tag	Sex	Date of birth	Date of vaccination	Age in days**	Time under close observation*	Evidence of vaccination remaining
362	F	Mar. 13	Mar. 27	14	2 months	Firm swelling in neck.
371	F	Mar. 29	Apr. 5	7	2 months	No swelling in neck.
373	F	Apr. 7	Apr. 18	11	2 months	No swelling in neck to be felt now.
374	F	Apr. 13	Apr. 18	5	2 months	Firm swelling in neck.
377	F	Apr. 18	Apr. 21	3	2 months	Firm swelling in neck.
379	F	Apr. 24	Apr. 28	4	2 months	No swelling to be felt.
381	F	Apr. 27	Apr. 28	1	2 months	Firm swelling.
382	M	May 25	May 29	4	2 months	Small firm swelling.
383	M	May 25	May 29	4	2 months	Firm swelling in neck.
384	F	May 29	May 29	..	2 months	Firm swelling in neck.
385	F	June 2	June 4	2	2 months	Relatively soft swelling.
387	M	June 14	June 30	16	2 months	Flat firm swelling.
388	F	June 21	June 30	9	2 months	Firm swelling.
389	M	June 23	June 30	7	2 months	Firm swelling.
390	F	June 27	June 30	3	2 months	Firm swelling.
391	M	July 12	July 29	17	2 months	Firm swelling.
392	M	July 13	July 29	16	2 months	Relatively soft swelling.
393	F	Aug. 8	Aug. 20	12	2 months	Large swelling.

* *Close observation means time during which records of temperature and weight were kept. The animals were otherwise under observation for six months at least.*
** *Age at vaccination.*

At the present time, we have records of numerous animals, which have shown no ill effects, as far as could be observed, from the vaccination, and of these many have received subsequent vaccinations.

Virulence

On September 28, 1925, 10 steers free from tuberculosis, as indicated by the use of tuberculin, and from a source free from tuberculosis, were vaccinated with 60 mg. of B.C.G. vaccine. One of these animals—No. 99—was slaughtered on December 30, 1925, and showed no evidence of tuberculosis.

TABLE III.

EXAMINATION OF STEER NO. 99—KILLED DECEMBER 30, 1925.

Tissue from dewlap—Mass of tissue 12 x 6 x 1 cm. In mass can be palpated three firm irregular nodules of the size of a bean.

Cut surfaces of nodules show reddish fibrous masses with no obvious caseous or purulent content.

Smears made from nodules of mass show a few distinctly staining acid-fast bacilli.

No evidence of tuberculosis of glands or organs on gross examination.

Two guinea pigs inoculated with material from nodule from dewlap December 31, died March 29, and April 1, respectively, from an epidemic disease among the guinea pigs. Autopsy showed no evidence of tuberculosis in glands or organs.

It should be added that with material cut during life from a B.C.G. vaccine nodule in three animals free from tuberculosis, we were not able to produce lesions in guinea pigs.

The remaining steers were killed on April 26, 1926, (seven months after vaccination) and showed no evidence of tuberculosis. Eight guinea pigs inoculated with glandular material from two of these animals—No. 94 and No. 100—showed no evidence of tuberculosis when examined from three to six months afterwards.

TABLE IV.

EXAMINATION OF REMAINING STEERS OF SERIES KILLED APRIL 29, 1926.

Steer No. 87—Well pronounced lump in dewlap, 3 cm., with no evidence of caseous or purulent content.
No gross evidence of tuberculosis in glands or organs.
Steer No. 96—Small nodule in dewlap, 1 cm.
Steer No. 97—Barely appreciable lump in dewlap.
Steer No. 88—Nodule in dewlap of moderate size, 2.5 cm. No obvious caseous or purulent contents.
Steer No. 98—Small nodule in dewlap, 1 cm.
Steer No. 94—Small nodule in dewlap, 1 cm.
Steer No. 95—No perceptible nodule in dewlap.
Steer No. 91—No perceptible nodule in dewlap.
Steer No. 100—No perceptible nodule in dewlap.
No evidence, gross or microscopic, in these animals of tuberculosis of glands or organs. Abscess of the liver present in steers Nos. 88, 95, 94. (So-called feed abscess).

GUINEA PIG INOCULATION

Steer	Guinea pigs	Gland	Date inoculated	Date killed	P.M. findings
No. 94	2	Mediastinal	Apr. 30	Sept. 24, 1926	Negative
	2	Mediastinal	Apr. 30	Sept. 9, 1926	Negative
	2	Bronchial	Apr. 30	Oct. 28, 1926	Negative
	2	Bronchial	Apr. 30	Aug. 17, 1926	Negative
No. 100	2	Mediastinal	Apr. 30	Sept. 9, 1926	Negative
	2	Mediastinal	Apr. 30	Sept. 24, 1926	Negative
	2	Bronchial	Apr. 30	Aug. 16, 1926	Negative
	2	Bronchial	Apr. 30	Oct. 28, 1926	Negative

We have thus no evidence to show that B.C.G. is pathogenic for bovines. The condition apparently remains local at the site of vaccination, and after seven months the glands in the neighbourhood and elsewhere are not involved. It must be stated as important that these animals were thoroughly isolated and had no opportunity of acquiring a virulent bacillus either naturally or artificially.

Immediate Contact Conditions

In this series of experiments animals were placed in contact with tuberculous animals immediately following subcutaneous vaccination.

In June, 1926, we reported the results of experiments in which out of 16 protected animals 75 per cent were free from tuberculosis macroscopically, while all controls showed evidence of disease (Group I). It was also reported that with two exceptions the type of disease in the vaccinated animals was more chronic and tended to calcification more than in the controls. These animals had been fed on mixed milk known to contain tubercle bacilli, and had been in contact since birth with tuberculous animals.

TABLE V.

GROUP I. — (A) NON-VACCINATED CONTROLS

Tag	Sex	Date of birth	Date of slaughter	Post-mortem findings
362	M	Jan. 31, 1925	Apr. 9, 1926	Generalized tuberculosis.
370	M	Mar. 27, 1925	Apr. 10, 1926	Very slight calcareous nodules in glands.
372	M	Apr. 5, 1925	Apr. 9, 1926	Calcareous nodules in glands.
375	M	Apr. 13, 1925	Apr. 10, 1926	Extensive caseous nodules in glands.
378	M	Aug. 18, 1925	Apr. 10, 1926	Extensive caseous nodules in glands.
380	M	Aug. 18, 1925	Apr. 10, 1926	Extensive caseous nodules in glands.
386	M	Aug. 18, 1925	Apr. 10, 1926	Calcareous glands.

In May, 1927, we had for experimental purposes a group of animals, (Group V) consisting of nine vaccinated animals which had been living in contact with tuberculous animals since birth, and four non-vaccinated controls which had existed under similar conditions.

These animals were slaughtered on May 18. Four only of the vaccinated animals were macroscopically free from tuberculosis. The controls—with one exception—showed active tuberculosis. In the vaccinated animals the lesions were mostly calcareous. The details are given in Table VII.

TABLE VI.

GROUP I. — (B) VACCINATED ANIMALS

Tag	Sex	Date of birth	Date of vaccination	Date killed	Post-mortem findings
381	F	Apr. 27–25	Apr. 28–25	Apr. 9–26	Pharyngeal glands slightly calcified.
382	M	May 25–25	May 29–25	Apr. 9–26	Pharyngeal post mediastinal and mesenteric calcareous.
384	F	May 29–25	May 29–25	Apr. 9–26	Portal, small calcareous nodule.
383	M	May 25–25	May 29–25	Apr. 9–26	No evidence of tuberculosis.
387	M	June 14–25	June 30–25	Apr. 9–26	No evidence of tuberculosis.
389	M	June 23–25	June 30–25	Apr. 9–26	No evidence of tuberculosis.
391	M	July 13–25	July 29–25	Apr. 9–26	No evidence of tuberculosis.
392	M	July 13–25	July 29–25	Apr. 9–26	No evidence of tuberculosis.
394	M	Sept. 5–25	Sept. 9–25	Apr. 9–26	No evidence of tuberculosis.
426	M	Feb. 5–26	Feb. 10–26	May 21–26	No evidence of tuberculosis.
425	M	Feb. 2–26	Feb. 4–26	May 20–26	No evidence of tuberculosis.
418	M	Jan. 19–26	Jan. 25–26	May 21–26	No evidence of tuberculosis.
417	M	Jan. 17–26	Jan. 20–26	May 21–26	No evidence of tuberculosis.
408	M	Dec. 31–25	Jan. 6–26	May 20–26	No evidence of tuberculosis.
397	M	Nov. 2–25	Nov. 12–25	May 20–26	No evidence of tuberculosis.
406	M	Dec. 29–25	Dec. 31–25	May 20–26	Bronchial gland extensively calcified.

NOTE—*Pigs inoculated with glandular material from Nos. 425, 418 and 417, did not show tuberculosis lesions at autopsy 5 months later.*

TABLE VII.

GROUP V. — (A) VACCINATED ANIMALS

Tag	Sex	Date of birth	Date of vaccination		Date killed	Post-mortem findings
			1st.	2nd.		
427	F	Feb. 8–26	10–2–26	19–2–27	18–5–27	No evidence of T. B.
428	M	Feb. 9–26	10–2–26	22–2–27	18–5–27	Lungs and glands involved.
433	M	Feb. 25–26	28–2–26	22–2–27	18–5–27	No evidence of T. B.
434	M	Mar. 1–26	11–3–26	22–2–27	18–5–27	Lungs and glands involved.
441	M	Mar. 29–26	5–4–26	5–4–27	18–5–27	Med-mediastinal calcareous.
443	M	Apr. 27–26	16–4–26	5–4–27	18–5–27	Bronchial and mediastinal calcareous.
450	M	May 23–26	26–5–26		18–5–27	No evidence of T. B.
452	M	July 20–26	26–7–26		18–5–27	No evidence of T. B.
454	M	July 20–26	26–7–26		18–5–27	Lungs (one spot) and bronchial glands show caseous spots.

GROUP V — (B) NON-VACCINATED CONTROLS

456	M	Aug. 22–26	Not vaccinated	18–5–27 18–5–27	Lungs, mediastinal and bronchial glands caseous.
457	M	Sept. 27–26	Not vaccinated	18–5–27	Lungs, mediastinal and bronchial glands caseous.
459	M	Oct. 28–26	Not vaccinated	18–5–27	Pharyngeal spreading.
	M	.....	Not vaccinated	18–5–27	Calcified glands.

TABLE VIII

MICROSCOPIC EXAMINATION OF THE LYMPH GLANDS OF THE ANIMALS IN GROUP V.

(A) VACCINATED ANIMALS.

Tag	Retroph.	Submax.	Portal	Mesenter.	Bronchial	Mediast.
427	Neg.	Neg.	Neg.	Neg.	One small but definite tubercle seen.	Neg.
428	Neg.	Neg.	Neg.	Neg.	Caseous lesions.	Caseous lesion in one node.
433	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.
434	Caseo-calcareous lesion.	Neg.	Neg.	Neg.	Extensive caseous lesions.	Extensive caseous and calcareous lesions in 2 of 3 glands.
441	Neg.	Neg.	Neg.	Neg.	Neg.	Calcareous lesion of one gland.
443	Neg.	Neg.	Neg.	Neg.	Calcareous lesion.	Extensive caseo-calcareous lesions in 3 of 4 glands.
450	Few small miliary foci.	Neg.	Neg.	Neg.	Neg.	Neg.
452	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.
454	Neg.	Neg.	Neg.	Neg.	Extensive caseo-calcareous lesion.	Neg.

(B) CONTROLS

456	Neg.	One small caseous focus	Neg.	Neg.	Caseous foci.	Caseous foci.
457	Neg.	Neg.	Neg.	Neg.	Extensive caseo-calcareous lesion.	Caseo-calcareous lesion in one gland.
459	Diffuse miliary foci.	Neg.	Neg.	Neg.	Caseous and miliary foci.	Neg.
...	Neg.	Neg.	Neg.	Calcareous lesion.	Neg.	Neg.

NOTE—*These results correspond to the microscopic findings with the exceptions that animal No. 427 (Vac.) shows a small lesion in one of the bronchial glands and animal No. 450 (Vac.) a few small miliary foci in one of the retropharyngeal glands.*

Delayed Contact Conditions

These animals were placed in contact with tuberculous animals only after two months of isolation subsequent to subcutaneous vaccination.

Group VI consisted of sixteen vaccinated animals and seven controls. These animals had been acquired as calves and vaccinated shortly after birth, fed on pasteurized milk for two months, and then placed in contact with a tuberculous herd and fed on milk containing tubercle bacilli. Here we find all the vaccinated animals free from tuberculosis macroscopically and microscopically, whereas six of the controls show caseous lesions. The details are given in Tables IX to XI (*B*).

TABLE IX.

GROUP VI. — VACCINATED ANIMALS

Tag	Sex	Date of vaccination	Date killed	Post-mortem findings
15	M	June 10–26	May 17–27	No evidence of tuberculosis.
17		June 10–26	May 17–27	No evidence of tuberculosis.
21	M	July 21–26	May 17–27	No evidence of tuberculosis.
22	M	June 16–26	May 17–27	No evidence of tuberculosis.
25	F	June 18–26	May 17–27	No evidence of tuberculosis.
29	F	June 23–26	May 17–27	No evidence of tuberculosis.
30	M	June 27–26	May 17–27	No evidence of tuberculosis.
33	F	July 1–26	May 17–27	No evidence of tuberculosis.
34	M	July 1–26	May 17–27	No evidence of tuberculosis.
501	F	July 30–26	May 17–27	No evidence of tuberculosis.
502		July 30–26	May 17–27	No evidence of tuberculosis.
503	M	July 31–26	May 17–27	No evidence of tuberculosis.
504		July 31–26	May 17–27	No evidence of tuberculosis.
505	M	Aug. 2–26	May 17–27	No evidence of tuberculosis.
507		Aug. 2–26	May 17–27	No evidence of tuberculosis.
508		Aug. 2–26	May 17–27	No evidence of tuberculosis.

TABLE X.

MICROSCOPIC EXAMINATION OF THE LYMPH GLANDS OF VACCINATED ANIMALS OF GROUP VI.

Tag	Retrophar.	Submax.	Portal	Mesenteric	Bronchial	Mediastinal Ant. Mid. Post.
15	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.
17	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.
21	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.
22	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.
25	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.
29	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.
30	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.
33	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.
34	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.
501	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.
502	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.
503	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.
504	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.
505	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.
507	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.
508	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.

TABLE XI (A).

GROUP VI — NON-VACCINATED CONTROLS

Tag	Sex	Date of vaccination	Date killed	Post-mortem findings
511		Not vaccinated	May 17–27	Caseous glands.
513		Not vaccinated	May 17–27	Caseous lungs and glands.
514	F	Not vaccinated	May 17–27	No evidence of tuberculosis.
512	F	Not vaccinated	May 17–27	Caseous lungs and glands.
516	M	Not vaccinated	May 17–27	Caseous lungs and glands.
519	F	Not vaccinated	May 17–27	Caseous lungs and glands.
520		Not vaccinated	May 17–27	Lungs, slight; glands slightly caseous.

TABLE XI (B).

MICROSCOPIC EXAMINATION OF THE LYMPH GLANDS OF THE UNVACCINATED CONTROLS OF GROUP VI.

Tag	Retrophar.	Submax.	Portal	Mesenteric	Bronchial	Mediastinal Ant. Mid. Post.
511	Neg.	Neg.	Neg.	Neg.	Caseous	Neg.
513	Neg.	Neg.	Neg.	Neg.	Neg.	Extensive diffuse caseous lesions in all three glands.
514	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.
512	Neg.	Neg.	Neg.	Neg.	Neg.	Extensive caseous lesions in one node.
516	Neg.	Neg.	Neg.	Neg.	Neg.	Extensive caseous-calcareous lesions in one gland.
519	Neg.	Neg.	Neg.	Neg.	Neg.	Caseous foci in one gland.
520	Neg.	Neg.	Neg.	Neg.	Few caseous foci	Numerous caseous foci in 2 or 3 glands.

NOTE—*These results correspond to the macroscopic findings.*

This might be considered a striking result. It will be observed that neither vaccinated animals nor controls were put in contact with tuberculous animals for a period of two months subsequent to vaccination. It would appear, therefore, that resistance in vaccinated animals is well developed two months after vaccination.

VACCINATION FOLLOWED BY INTRAVENOUS INOCULATION WITH VIRULENT BACILLI, 1926

On the first of June, 1926, it was reported that we had under investigation 25 animals, which had been inoculated intravenously on February 29, 1926, with 5 mg. of a strain of *B. tuberculosis* (Vallée) obtained fron Calmette. (The growth was one month old on Petroff's medium with the material suspended in saline, the inoculation being made into the jugular vein). The animals

included ten (Group II) from a tuberculosis-free source, which had not been in contact with tuberculous animals, and which had previously been given, subcutaneously, into the dewlap, 60 mg. of B.C.G. vaccine, (eight on October 7, 1925, one on May 17, 1925 and one on May 20, 1925) also nine unvaccinated controls (Group III) from a tuberculosis-free source, which did not react to tuberculin on February 5, 1926.

Group II, Vaccinated Animals

Ten vaccinated animals (with B.C.G., as above) were inoculated intravenously on February 5, 1926, with 5 mg. of a month-old culture of *B. tuberculosis* (Vallée) and were killed on September 21, 1926. Seven months after inoculation these showed six animals free from macroscopic tuberculosis lesions, one doubtful, two with glandular lesions and one with a heavy pleuritic, glandular and pulmonary involvement, as noted in Table XII.

TABLE XII.

VACCINATED ANIMALS INOCULATED WITH *B. tuberculosis* VALLEE

Tag	Date of vaccination	Date of inoculation	Date killed	Post-mortem findings
58	Oct. 7–25	Feb. 25–26	Sept. 21–26	No evidence of tuberculosis.
59	Oct. 7–25	Feb. 25–26	Sept. 21–26	No evidence of tuberculosis.
60	Oct. 7–25	Feb. 25–26	Sept. 21–26	No evidence of tuberculosis.
61	Oct. 7–25	Feb. 25–26	Sept. 21–26	Localized (calcareous) glandular tuberculosis.
62	Oct. 7–25	Feb. 25–26	Sept. 21–26	One caseous nodule in right lung, a few pleuritic adhesions and a very large (non-tubercular) abscess in the liver.
63	Oct. 7–25	Feb. 25–26	Sept. 21–26	No evidence of tuberculosis.
64	Oct. 7–25	Feb. 25–26	Sept. 21–26	No evidence of tuberculosis.
66	Oct. 7–25	Feb. 25–26	Sept. 21–26	Lungs, pleura and mediastinal glands all affected.
303	May 17–25	Feb. 25–26	Sept. 21–26	Very slight (calcareous) glandular tuberculosis.
314	May 17–25	Feb. 25–26	Sept. 21–26	No evidence of tuberculosis.

Of 63 guinea pigs, inoculated with glandular and other material from these animals on September 23, 1926, all, (with the exception of those inoculated with material from animal No. 63,) showed evidence, subsequently, of tuberculosis at autopsy, as a result of infection with the virulent organism used for inoculation and still present in the tissues of the animals.

Group III, Unvaccinated Animals.

Nine unvaccinated animals from a tuberculosis-free source were inoculated intravenously on the 25th of February, 1926, with 5 mg. of a month-old culture of *B. tuberculosis* (Vallée), after giving a negative tuberculin

test on Feb. 5, 1926. Of these animals No. 69 died on the 30th of April showing generalized tuberculosis at autopsy, while No. 70 died on the 10th of September, post-mortem examination revealing generalized tuberculosis. Of the remaining seven animals, one showed no macroscopic evidence of tuberculosis, five showed localized glandular tuberculosis, and one extensive pleuritic involvement as well as glandular tuberculosis, as noted in Table XIII.

TABLE XIII.

UNVACCINATED CONTROLS INOCULATED WITH *B. tuberculosis* VALLEE

Tag	Not vaccinated	Date of inoculation	Date killed	Post-mortem findings
65	"	25–3–26	21–9–26	Pleura showed adhesions and extensive calcareous concretions on both sides with slight calcareous changes in the bronchial and mediastinal glands.
73	"	25–3–26	21–9–26	Slight calcareous changes in mediastinal glands and an old (healed) broncho-pneumonia.
75	"	25–3–26	22–9–26	Localized (calcareous) glandular infection.
102	"	25–3–26	22–9–26	Slight localized glandular infection.
103	"	25–3–26	22–9–26	Localized glandular infection with pericardial and peritoneal adhesions.
316	"	25–3–26	22–9–26	No evidence of tuberculosis.
345	"	25–3–26	22–9–26	Localized glandular infection.
69	"	25–3–26	Died 30–4–26	Generalized tuberculosis.
70	"	25–3–26	Died 10–9–26	Generalized tuberculosis.

These results indicate that the strain of *B. tuberculosis* (Vallée) used, according to the conditions of the experiment, was not capable of causing generalized tuberculosis in even the unprotected animals (growth possibly too old), although two of them died before September 21, 1926, (No. 69 of Group III on April 30 and No. 70 of Group III on September 10) showing advanced tuberculous lesions at autopsy. On the other hand, they indicate resistance in the vaccinated (B.C.G.) animals, for here we find 60 per cent of the animals without evident macroscopic lesions at autopsy. (The unvaccinated showed 18 per cent). These experimental results might have been much clearer, as regards contrast, had the infecting organism used had greater invasive powers. The guinea-pig results also indicate resistance, in that they show the presence of virulent organisms, without macroscopic lesions. It is important to remember, in this connection, that with tuberculosis-free animals inoculated with B.C.G. vaccine we have not been able to produce lesions in guinea pigs with glandular material taken at autopsy nor with tissue showing tubercle bacilli from the site of inoculation, cut out during life or taken at autopsy.

In consideration of these results we are of the opinion that in such an experiment, where virulent bacilli (very virulent for the guinea pig) have been used, macroscopic findings are a most valuable evidence of resistance, and that positive guinea-pig findings indicate the presence of virulent bacilli, probably held in check in the vaccinated animals.

Experiments I, II and III, 1927-28

In the spring of the year 1927, we started the purchase of new-born calves for the continuation of our work, and for the repetition of experiments already reported to the National Research Council.

These calves were purchased between the months of March and September, the animals being secured as soon after birth as possible, and removed to a new barn not previously used. Some of them were immediately vaccinated subcutaneously in the dewlap with 60 mg. moist B.C.G. 21 days old, grown on glycerine potato at 38 deg. C., and all were fed on pasteurized milk for a period of two months. By this means we collected during the time mentioned 112 calves, so that by the first of September, 1927, we had at our disposal 62 vaccinated calves and 50 unvaccinated controls, all of which had been removed from possible sources of tuberculous infection shortly after birth, and subsequently fed on pasteurized milk for a period of two months. For the purpose of experiment these calves were then divided into groups.

Group X

Twelve vaccinated animals were chosen for the purpose of repeating our previous experiment reported May 27, 1927, testing the possible virulence of B.C.G. for new-born calves.

Group Y

Fifty calves, consisting of 25 vaccinated animals and 25 controls, were held for an inoculation experiment similar to that reported by us on May 27, 1927.

Group Z

The remaining 50, consisting of 25 vaccinated and 25 non-vaccinated animals, were sent to contact with a tuberculous herd, and to be fed daily on a certain quantity of milk from that herd, a repetition of our experiment communicated to the Council on May 27, 1927.

Experiment No. 1, 1927-28. (Group X). Virulence.

These animals were reduced to ten, two having died while quite young of disease other than tuberculosis. They were slaughtered April 2, 1928, one year after vaccination, and were carefully examined for the evidences of tuberculosis in the presence of several competent observers. Macroscopically, at post-mortem examination no evidence of tuberculous lesions could be discovered, and it was in some cases somewhat difficult to determine the original point of vaccination. Material taken from the lymph glands of each of these animals for histological examination and inoculation into guinea pigs gave negative results. The results are given in Table XIV.

TABLE XIV.

EXPERIMENT TO DETERMINE VIRULENCE OF B.C.G.

Tag	Date of birth	Date of vaccination	Date of slaughter	Post-mortem
48	Mar. 21–27	Mar. 23–27	Apr. 2–28	No macroscopic evidence of tuberculosis.
49	Mar. 24–27	Mar. 25–27	Apr. 2–28	No macroscopic evidence of tuberculosis.
50	Mar. 25–27	Mar. 26–27	Apr. 2–28	No macroscopic evidence of tuberculosis.
51	Mar. 26–27	Mar. 26–27	Apr. 2–28	No macroscopic evidence of tuberculosis.
54	Mar. 26–27	Mar. 28–27	Apr. 2–28	No macroscopic evidence of tuberculosis.
56	Mar. 30–27	Mar. 30–27	Apr. 2–28	No macroscopic evidence of tuberculosis.
101	Mar. 30	Mar. 30–27	Apr. 2–28	No macroscopic evidence of tuberculosis.
104	Apr. 4	Apr. 4–27	Apr. 2–28	No macroscopic evidence of tuberculosis.
108	Apr. 6–27	Apr. 6–27	Apr. 2–28	No macroscopic evidence of tuberculosis.
110	Apr. 5–27	Apr. 8–27	Apr. 2–28	No macroscopic evidence of tuberculosis.

TABLE XV.

* MICROSCOPIC EXAMINATION OF THE LYMPH GLANDS OF THE ANIMALS IN GROUP X.

Animal	Retroph.	Submax.	Portal	Mesanter.	Bronch.	Mediast.	Prescap.	Cervical
49	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.
56	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.
51	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.
50	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.
110	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.
54	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.
104	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.
108	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.
48	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.
101	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.

Dewlap No. 54—Extensive caseo-calcareous lesion with fibrosis and chronic inflammatory cell infiltration.

Dewlap No. 108—Tubercles with fibrosis and calcification.

Dewlap No. 101—Fibrosis with hyalin change. Areas of small round cell infiltration. No tubercles or caseation.

* NOTE—*The lymph nodes of the above animals show no evidence of tuberculosis microscopically.*

TABLE XVI

RESULTS OF GROSS AND MICROSCOPICAL EXAMINATION OF GUINEA PIGS INOCULATED WITH MATERIAL* FROM ANIMALS OF EXPERIMENT No. I, 1927.

Inoc. April 2, 1928.

No. 49.	*Pig 1.*	Died June 15, 1928. No evidence of tuberculosis grossly or microscopically.
	Pig 2.	Killed June 18, 1928. One enlarged lymph node of neck with suppurative focus in centre, smear from which shows gram-positive cocci. One small healed pyaemic focus in spleen microscopically. No gross or microscopic evidence of tuberculosis.
	Pig 3.	Killed June 18, 1928. No gross or microscopic evidence of tuberculosis.
	Pig 4.	Killed June 18, 1928. No gross or microscopic evidence of tuberculosis.
No. 56.	*Pig 5.*	Died April 4, 1928. No autopsy.
	Pig 6.	Died May 11, 1928. Acute purulent bronchitis. Broncho-pneumonia. Acute hyperlastic splenitis. No evidence of tuberculosis grossly or microscopically.
	Pig 7.	Killed June 18, 1928. No gross or microscopic evidence of tuberculosis.
	Pig 8.	Died April 4, 1928. No autopsy.
No. 51.	*Pig 9.*	Killed June 6, 1928. Pustular enlargement submental lymph node, pyogenic abscess microscopically. No gross or microscopic evidence of tuberculosis.
	Pig 10.	Died April 7. No autopsy.
	Pig 11.	Killed June 21, 1928. Pregnant. No gross or microscopic evidence of tuberculosis.
	Pig 12.	Died April 5. No autopsy.
No. 50.	*Pig 13.*	Killed June 11, 1928. No gross or microscopic evidence of tuberculosis.
	Pig 14.	Killed June 7, 1928. Old inflammatory exudate at site of inoculation microscopically. No gross or microscopic evidence of tuberculosis.
	Pig 15.	Killed June 7, 1928. No gross or microscopic evidence of tuberculosis.
	Pig 16.	Killed June 11, 1928. No gross or microscopic evidence of tuberculosis.
No. 110.	*Pig 17.*	Killed June 4, 1928. Recently pregnant. No gross or microscopic evidence of tuberculosis.
	Pig 18.	Killed June 28, 1928. No gross or microscopic evidence of tuberculosis.
	Pig 19.	Killed Aug. 20, 1928. No gross or microscopic evidence of tuberculosis.
	Pig 20.	Died June 3, 1928. Acute enteritis. No gross or microscopic evidence of tuberculosis.
No. 54.	*Pig 21.*	Died May 8, 1928. Ulcerative lesion at site of inoculation. Emaciation. No involvement of glands or organs. No gross or microscopic evidence of tuberculosis.
	Pig 21A.	Reinoculation from ulcerative area at site of inoculation. Showed diffuse suppurative skin lesion around site of inoculation, and acute osteomyelitis, pyogenic infection. No evidence of tuberculosis in organs or glands.
	Pig 22.	Died April 23, 1928. Purulent exudate at site of inoculation. Smears showed streptococci but no tubercle bacilli. Acute congestion of lungs. No gross or microscopic evidence of tuberculosis. Streptococcus infection.
	Pig 23.	Killed June 28, 1928. No gross or microscopic evidence of tuberculosis.
	Pig 24.	Killed June 28, 1928. No gross or microscopic evidence of tuberculosis.
No. 104.	*Pig 25.*	Killed July 5, 1928. No gross or microscopic evidence of tuberculosis.
	Pig 26.	Died May 18, 1928. Purulent mass at site of inoculation. Acute suppurative peritonitis, pleuritis and pericarditis. Smears showed streptococci but no tubercle bacilli. Streptococcic septicaemia.
	Pig 27.	Killed June 30, 1928. No gross or microscopic evidence of tuberculosis.
	Pig 28.	Killed June 30, 1928. No gross or microscopic evicence of tuberculosis.

* *Material from : retroperitoneal, submaxiliary, portal, mesenteric, bronchial, mediastinal, prescapular and cervical lymph glands.*

TABLE XVI, CONTINUED

RESULTS OF GROSS AND MICROSCOPICAL EXAMINATION OF GUINEA PIGS INOCULATED WITH MATERIAL* FROM ANIMALS OF EXPERIMENT No I, 1927.

Inoc. April 2, 1928.

No. 108.	*Pig 29.*	Killed July 5, 1928. No gross or microscopic evidence of tuberculosis.
	Pig 30.	Killed July 5, 1928. Small area pleuritic thickening, subacute pleuritis microscopically. No gross or microscopic evidence of tuberculosis.
	Pig 31.	Killed June 30, 1928. Large submental gland with purulent centre. No gross or microscopic evidence of tuberculosis.
	Pig 32.	Died May 30, 1928. Recently pregnant. Haemorrhagic lesion of intestines and mesenteric lymph nodes. No gross or microscopic evidence of tuberculosis. Streptococcic infection.
No. 48.	*Pig 33.*	Killed July 9. No gross or microscopic evidence of tuberculosis.
	Pig 34.	Killed Aug. 20, 1928. No gross or microscopic evidence of tuberculosis.
	Pig 35.	Killed July 10, 1928. No gross or microscopic evidence of tuberculosis.
	Pig 36.	Killed July 14, 1928. No gross or microscopic evidence of tuberculosis.
No. 101.	*Pig 37.*	Killed July 9, 1928. No gross or microscopic evidence of tuberculosis.
	Pig 38.	Killed July 9, 1928. No gross or microscopic evidence of tuberculosis.
	Pig 39.	Killed May 21, 1928. No gross or microscopic evidence of tuberculosis.
	Pig 40.	Killed July 7, 1928. No gross or microscopic evidence of tuberculosis.
	Material from dewlap	
No. 54.	*Pig 41.*	Died May 13, 1928. Mass of purulent material at site of inoculation. Acute broncho-pneumonia, purulent bronchitis, acute cloudy swelling of liver. Gram positive cocci in pus from site of inoculation. No gross or microscopic evidence of tuberculosis.
	Pig 42.	Killed August 20, 1928. No gross or microscopic evidence of tuberculosis.
No. 108.	*Pig 43.*	Killed June 21, 1928. No gross or microscopic evidence of tuberculosis.
	Pig 44.	Killed June 21, 1928. No gross or microscopic evidence of tuberculosis.

* *Material from : retroperitoneal, submaxiliary, portal, mesenteric, bronchial, mediastinal, prescapular and cervical lymph glands.*

NOTE—*The suppurative lymph node of neck found in pigs Nos. 2, No. 9, No. 31, is the result of bleeding these pigs from jugular vein of neck to obtain complement.*

This experiment, taken in conjunction with our other observations, indicates that the vaccine is harmless when exhibited to young calves subcutaneously, and under the conditions of the experiment. After a period of one year we have failed to find evidence of tuberculous lesions either by microscopic examination or by the injection of glandular material into guinea pigs.

The question of the virulence of B.C.G. with respect to guinea pigs is somewhat confused, owing to the conflicting results of various workers. Some hold that lesions in guinea pigs after a heavy inoculation, subcutaneous or intraperitoneal, are transient, appear early, and finally disappear (1, 2, 3, 4, 5), while others hold that subsequently the bacillus may finally produce progressive lesions (6, 7, 8, 9, 10, 11).

Petroff (9, 10) has reported that he is able to divide B.C.G. into two strains, one of which is virulent for guinea pigs, while Calmette (1), on the other hand, reports that he is unable to confirm Petroff's results. We think it is clear that B.C.G. in large doses, particularly intraperitoneally, is capable of producing transient lesions, but we are unable, at the present time, on account of

the conflicting evidence, to quite make up our minds with respect to the degree of virulence to these animals (11). With respect to rabbits, we have information which leads us to suspect that the organism may possibly be capable of producing tuberculous lesions in these animals, but that, nevertheless, tuberculous tissue from such infected rabbits is incapable of infecting guinea pigs.

With bovines, we conclude from this experiment coupled with our experiment reported in May 1927, and other experimental results, that B.C.G. is not capable, apparently, of producing demonstrable lesions other than at the point of vaccination—*one year after vaccination*—and that there are no lesions, *macroscopic or microscopic*, in the glands draining the area of vaccination or elsewhere.

We think our guinea-pig inoculations indicate that the virulence of B.C.G. is not increased for these animals by a year's sojourn in the calf. In further support of this statement, as already reported to the National Research Council, 14 guinea pigs were inoculated with material containing acid-fast bacilli from the dewlap lesions of 5 vaccinated animals, and, at autopsy, after a period varying from 2 to 4 months, there were no demonstrable tuberculous lesions. We also conclude that such subcutaneous vaccinations in calves do not appear to interfere with normal development or condition within the time limit of our experiment.

TABLE XVII

FURTHER INOCULATION OF GUINEA PIGS WITH LESIONS FROM THE DEWLAP

Animal	Time elapsed since vac.	Acid-fast bac. present	No. of G. P. inoculations	Date of inoculations	Date of P.M. examination	Time elapsed	Results
Steer 99 1925	3 mos.	Yes	2	Dec. 31–1925	Apr. 1–26	3 mos.	Negative
Calf 310 1925	9 mos.	Yes	4	Jan. 20–1926	Mar. 17–26	2 mos.	Negative
					Apr. 11–26	10 weeks	Negative
					Apr. 23–26	3 mos.	Negative
					Apr. 27–26	4 mos.	Negative
Calf 312	8 mos.	Yes	4	Jan. 20–1926	Mar. 21–26	2 mos.	Negative
					Mar. 21–26	2 mos.	Negative
					Mar. 23–26	2 mos.	Negative
					Apr. 22–26	3 mos.	Negative
No. 54 1927–28	1 year	Yes	2	Apr. 2–1928	May 13–28	5 weeks	Negative
					Aug. 20–28	4½ mos.	Negative
No. 108 1927–28	1 year	Not examined microscopically	2	Apr. 2–1928	June 21–28	2½ mos.	Negative
					June 21–28	2½ mos.	Negative
Total			14				14

VACCINATION FOLLOWED BY INTRAVENOUS INOCULATION OF VIRULENT BACILLI, 1927

Experiment No. II, 1927-28. (*Group Y*)

On March 12, in some cases nearly a year after vaccination, the members of this group were given an intravenous injection of 5 mg. (moist) of a young culture (3 weeks) of *B. tuberculosis* (Vallée) grown on potato. Six

of the non-vaccinated controls died of tuberculosis or were slaughtered before impending death, and one of the vaccinated animals died of tuberculosis. The details available are shown in Table XVIII.

TABLE XVIII.

UNVACCINATED CONTROLS INOCULATED WITH *B. tuberculosis* VALLÉE

Tag	Date of birth	Date of inoculation	Date of slaughter or death	Post-mortem findings
178	14–5–27	Mar. 12–28	(1) Died 16–5–28	Generalized tuberculosis.
176	11–5–27	Mar. 12–28	Killed 25–6–28	Lungs, bronchial and mediastinal caseous.
175	11–5–27	Mar. 12–28	Killed 25–6–28	Lungs, bronchial and mediastinal caseous.
174	11–5–27	Mar. 12–28	Killed 25–6–28	Lungs, scattered tubercles.*
173	11–5–27	Mar. 12–28	Killed 25–6–28	Lungs, bronchial and mediastinal caseous.
172	11–5–27	Mar. 12–28	(2) Died 20–5–28	Generalized tuberculosis.
171	10–5–27	Mar. 12–28	(3) Died 2–5–28	Generalized tuberculosis.
170	9–5–27	Mar. 12–28	Killed 25–6–28	Caseous pharyngeal, bronchial and mediastinal.
169	6–5–27	Mar. 12–28	Killed 25–6–28	Generalized tuberculosis.
167	8–5–27	Mar. 12–28	Killed 25–6–28	Nodules in lung, bronchial.
165	6–5–27	Mar. 12–28	Killed 25–6–28	Lungs caseous nodules, bronchial and mediastinal caseous.
164	5–5–27	Mar. 12–28	Killed 25–6–28	Generalized tuberculosis.
163	3–5–27	Mar. 12–28	(4) Died 19–5–28	Generalized tuberculosis.
161	27–4–27	Mar. 12–28	Killed 25–6–28	Lungs tuberculous.
160	29–4–27	Mar. 12–28	Killed 25–6–28	Lungs, bronchial, mediastinal and mesenteric caseous.
159	23–4–27	Mar. 12–28	Killed 25–6–28	Lungs, bronchial, mediastinal and mesenteric caseous.
158	22–4–27	Mar. 12–28	Killed 25–6–28	Lungs, bronchial, mediastinal and mesenteric caseous.
157	21–4–27	Mar. 12–28	(5) Died 14–5–28	Generalized tuberculosis.
155	22–4–27	Mar. 12–28	Killed 27–6–28	Lungs caseous lesions, bronchial caseous calcareous, mediastinal caseous.
154	10–4–27	Mar. 12–28	Killed 27–6–28	Bronchial and mediastinal caseous.
153	5–4–27	Mar. 12–28	Killed 27–6–28	Lungs, bronchial and mediastinal caseous.
152	6–4–27	Mar. 12–28	Killed 27–6–28	Lungs caseous.
151	10–4–27	Mar. 12–28	(6) Died 12–5–28	Generalized tuberculosis.

* *Macroscopic findings with respect to Animal No. 174 not confirmed microscopically.*

NOTE—*Two calves originally included in this group died of disease other than tuberculosis early in the experiment.*

Six of the animals in Exp. II died of generalized tuberculosis within sixty day of inoculation with *B. tuberculosis* (Vallée), and, of the remaining seventeen, sixteen showed caseous lesions at autopsy confirmed microscopically—that is to say 95 per cent were tuberculous, showing active lesions.

TABLE XIX.

MICROSCOPIC EXAMINATION OF THE LYMPH GLANDS OF CONTROLS IN EXPERIMENT II.

Animal	Retrophar.	Submax.	Mesenter.	Portal	Cervical	Bronchial	Prescap.	Mediastinal	Lung
178	Scattered tubercles slight caseation	Scattered tubercles	Scattered tubercles and caseous foci.	Scattered tubercles.	Neg.	Scattered tubercles and areas of caseation.	Neg.	Extensive caseous lesions in all three glands	Caseous pneumonia
176	Neg.	Neg.	Neg.	Neg.	Neg.	Caseo-calcareous lesions.	Neg.	Caseo-calcarous lesions in two glands, scattered tubercles on one gland.	No lesions in material sectioned.
175	Neg.	Neg.	Neg.	Scattered small tubercles.	Neg.	Caseo-calcareous lesions.	Neg.	Extensive caseo-calcareous lesions in all three nodes.	Conglom. tubercles.
174	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Small scattered areas of broncho-pneumonia. No definite tuberculous lesions.
173	Neg.	Neg.	Neg.	Scattered tubercles.	Scattered tubercles.	Numerous tuberculous foci.	Scattered tubercles.	Numerous tuberculous areas in all three nodes.	.....
172	.....	.....	Neg.	Extensive caseous lesions.	Neg.	Scattered tubercles.	Diffuse caseous lesions with small areas of calcification.	Extensive caseo-calcarous lesions in all three nodes.	Extensive caseous lesions.
171	Few scattered tubercles.	Neg.	Scattered tubercles.	Scattered conglomerate tubercles	Neg.	Extensive caseous lesions.	Neg.	Extensive caseous lesions in all three nodes.	Broncho-pneumonia.

TABLE XIX, CONTINUED

MICROSCOPIC EXAMINATION OF THE LYMPH GLANDS OF CONTROLS IN EXPERIMENT II.

Animal	Retrophar.	Submax.	Mesenter.	Portal	Cervical	Bronchial	Prescap.	Mediastinal	Lung
170	Neg.	Neg.	Neg.	Scattered tubercles.	Neg.	Caseo-calcareous lesions.	Neg.	Caseo-calcareous lesions in one node—other two nodes show numerous tubercles.	.....
169	Few scattered tubercles.	Neg.	Neg.	Scattered conglomerate tubercles.	Neg.	Conglomerate tubercles.	Neg.	Numerous tuberculous areas in all three nodes.	Pneumonic process, probably tuberculous.
167	Neg.	Neg.	Neg.	One small tubercle seen in one section.	Neg.	Few scattered miliary tubercles.	Neg.	Scattered miliary foci in all three nodes.	Small tuberculous foci.
165	Neg.	Neg.	Neg.	Few scattered tubercles.	Neg.	Numerous conglomerate tubercles.	Neg.	Diffuse caseous lesions in one node—scattered tubercles in two nodes.	.....
164	Neg.	Neg.	Scattered tubercles.	Extensive caseous lesion numerous tubercles.	Neg.	Extensive caseo-calcareous lesions.	Neg.	Extensive caseo-calcareous lesions in all three nodes.	.....
163	Neg.	Neg.	Extensive caseous lesions.	Extensive caseous lesions.	Neg.	Extensive caseo-calcareous lesions.	Extensive caseous lesions.	Extensive caseo-calcareous lesions in all three nodes.	Numerous caseous lesions.
161	Neg.	Neg.	Neg.	Small cal-calcareous caseous focus. Few scattered tubercles.	Neg.	Neg.	Neg.	Scattered conglomerate and small tubercles in all three nodes.	Neg.

TABLE XIX, CONTINUED

MICROSCOPIC EXAMINATION OF THE LYMPH GLANDS OF CONTROLS IN EXPERIMENT II.

Animal	Retrophar.	Submax.	Mesenter.	Portal	Cervical	Bronchial	Prescap.	Mediastinal	Lung
160	Neg.	Neg.	Neg.	Neg.	Neg.	Extensive caseo-calcareous lesions.	Neg.	Extensive caseo-calcareous lesions. in all three nodes.	.....
159	Neg.	Neg.	Neg.	Few small tubercles at periphery of node.	Neg.	Extensive caseo-calcareous lesions.	Neg.	Extensive caseo-calcareous lesions in two nodes, numerous conglomerate tubercles in one node.	.....
158	Neg.	Neg.	No tissue.	Neg.	Neg.	Extensive caseo-calcareous lesions.	Neg.	Extensive caseo-calcareous lesions in all three nodes.	Few small tubercles.
157	Neg.	Neg.	Numerous conglomerate tubercles with caseation.	Neg.	No tissue.	Extensive caseous areas.	Numerous caseous conglomerate tubercles.	Extensive caseous neurosis in all three nodes.	Extensive tuberculous lesions.
154	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Scattered caseo-calcareous lesions in one node—conglomerate tubercles in other two nodes.	.....
153	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Calcareous lesions in one node—conglomerate tubercles in other two nodes.	.....

TABLE XIX, CONTINUED

MICROSCOPIC EXAMINATION OF THE LYMPH GLANDS OF CONTROLS IN EXPERIMENT II.

Animal	Retrophar.	Submax.	Mesenter.	Portal	Cervical	Bronchial	Prescap.	Mediastinal	Lung
152	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Scattered conglomerate and solitary tubercles in two of three nodes.	.....
151	Neg.	Neg.	Scattered conglomerate and solitary tubercles.	Numerous conconglomerate caseous foci and solitary tubercles.	Neg.	Extensive caseo-calcareous lesions.	Neg.	Extensive caseo-calcareous lesions in all three nodes.	Diffuse caseous pneumonia.
155	Neg.	Neg.	Neg.	Neg.	Neg.	Diffuse caseo-calcareous lesions.	Neg.	Diffuse caseo-calcareous in all three nodes.	Caseous lesions.

TABLE XX

EXPERIMENT II, 1927, VACCINATED ANIMALS

Tag	Date of birth	Date of vaccination	Date of inoculation	Date of slaughter	P. M. findings
145	10–6–27	13–6–27	12–3–28	27–6–28	No evidence of T. B.
144	9–6–27	9–6–27	12–3–28	Died 5–5–28	Generalized T. B.
143	8–6–27	9–6–27	12–3–28	25–6–28	No evidence of T. B.
142	8–6–27	9–6–27	12–3–28	25–6–28	Small healed tubercles in lungs-3 bronchial-1 mediastinal middle.
140	8–6–27	9–6–27	12–3–28	25–6–28	One small tubercle healed in lung.
139	5–6–27	7–6–27	12–3–28	25–6–28	No evidence of T. B.
137	3–6–27	4–6–27	12–3–28	25–6–28	No evidence of T. B.
135	24–4–27	26–4–27	12–3–28	25–6–28	No evidence of T. B.
134	16–4–27	23–4–27	12–3–28	25–6–28	No evidence of T. B.
133	19–4–27	23–4–27	12–3–28	25–6–28	No evidence of T. B.
132	19–4–27	23–4–27	12–3–28	25–6–28	No evidence of T. B.
126	19–4–27	19–4–27	12–3–28	27–6–28	No evidence of T. B.
124	18–4–27	19–4–27	12–3–28	25–6–28	No evidence of T. B.
123	19–4–27	19–4–27	12–3–28	25–6–28	No evidence of T. B.
122	18–4–27	19–4–27	12–3–28	25–6–28	No evidence of T. B.
121	15–4–27	18–4–27	13–3–28	25–6–28	No evidence of T. B.
120	15–4–27	18–4–27	12–3–28	25–6–28	No evidence of T. B.
119	12–4–27	16–4–27	12–3–28	27–6–28	Post mediastinal one calcareous nodule.
118	15–4–27	15–4–27	12–3–28	27–6–28	No evidence of T. B.
114	12–4–27	12–4–27	12–3–28	27–6–28	No evidence of T. B.
113	10–4–27	11–4–27	12–3–28	25–6–28	No evidence of T. B.
111	6–4–27	8–4–27	12–3–28	25–6–28	One small healed tubercle-lungs.
107	6–4–27	6–4–27	12–3–28	27–6–28	No evidence of T. B.
53	25–3–27	28–4–27	12–3–28	25–6–28	No evidence of T. B.
52	24–3–27	28–4–27	12–3–28	25–6–28	No evidence of T. B.

Out of the 25 animals, one died on May 5, of generalized tuberculosis, and, of the others, three showed one healed or calcareous lesion each and one showed five healing or calcareous lesions, in marked contrast to the nature of the lesions in the controls. That is to say, 20 per cent showed tuberculous lesions of a sort, and 80 per cent were macroscopically free therefrom. The nature of the lesions, healing and calcareous as contrasted with the caseous active lesions in the controls, is important and quite marked. All of these animals when slaughtered could have been passed for food.

TABLE XXI.

MICROSCOPIC EXAMINATION OF VACCINATED ANIMALS IN EXPERIMENT II.

Animal	Retroph.	Submax.	Mesenter.	Portal	Cervical	Bronch.	Prescap.	Mediast.	Lung
145	Neg.	Neg.	Neg.	Neg.	Neg.	Numerous conglomerate tubercles.	Neg.	Scattered conglomerate and solitary tubercles in all three nodes.	.....
144	Numerous conglom. tubercles.	Neg.	Neg.	Scattered conglomerate tubercles.	Neg.	Extensive caseocal. lesions.	No tissue.	Extensive caseocal. lesions in all three nodes.	Numerous caseous lesions.
143	Neg.	Neg.	Neg.	Neg.	Neg.	Few scattered tubercles.	Neg.	Scattered conglomerate and solitary tubercles in all three nodes.	.....
142	Neg.	Neg.	Neg.	Neg.	Neg.	Numerous conglom. tubercles, (encapsulated), slight caseation.	Neg.	Scattered conglom. tubercles in all three nodes.	Encap. caseous lesion.
140	Neg.	Neg.	Neg.	Neg.	Neg.	Few conglom. tubercles.	Neg.	Few tubercles in one of three nodes.	Bronchopneum. lesion.
139	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Few solitary tubercles in one of three nodes.	.....
137	Neg.	Neg.	Neg.	Neg.	Neg.	Few conglom. tubercles.	Neg.	Scattered conglom. tubercles in two of three nodes.	.....
135	Neg.	Neg.	Few scattered solitary tubercles.	Few scattered solitary tubercles.	No tissue .	Neg.	Neg.	Few scattered conglom. tubercles in all three nodes.	.....

TABLE XXI, CONTINUED

MICROSCOPIC EXAMINATION OF VACCINATED ANIMALS IN EXPERIMENT II.

Animal	Retroph.	Submax.	Mesenter.	Portal	Cervical	Bronch.	Prescap.	Mediast.	Lung
134	Neg.	Neg.	Neg.	Neg.	Neg.	Few scattered tubercles.	Neg.	Few scattered tubercles in all three nodes.	.....
133	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	.....
132	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Few scattered solitary tubercles in one of three nodes.	.....
126	Neg.	Neg.	Neg.	Neg.	Neg.	Few scattered conglomerate tubercles.	Neg.	Scattered conglom. tubercles in two or three nodes.	.....
124	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Few scattered conglom. and solitary tubercles in one node.	.....
123	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Few scattered conglomerate tubercles in all three nodes.	.....
122	Neg.	Neg.	Neg.	Neg.	Few scattered conglom. tubercles.	Few scattered conglom. tubercles.	Neg.	Few scattered conglom. tubercles in two of three nodes.	Encap. fibroid tubercles with slight caseation.
121	Neg.	Neg.	Few scatteted conglom. tubercles.	Neg.	Neg.	No tissue.	Neg.	Scattered conglom. tubercles in all three nodes.	.....

TABLE XXI, CONTINUED

MICROSCOPIC EXAMINATION OF VACCINATED ANIMALS IN EXPERIMENT II.

Animal	Retroph.	Submax.	Mesenter.	Portal	Cervical	Bronch.	Prescap.	Mediast.	Lung
120	Neg.	Neg.	Neg.	Neg.	Few scattered conglom. tubercles.	Few scattered conglom. tubercles.	Neg.	Several pinhead caseous areas in one node, scattered conglom. tubercles in other two.	.....
119	Neg.	Neg.	Neg.	Neg.	Neg.	Few scattered conglom. tubercles.	Neg.	Encap. caseocal. lesion in one, scattered tubercles in one, neg. one.	.....
118	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	.....
114	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	.....
113	Neg.	Neg.	Neg.	Few scattered conglom. tubercles.	Neg.	Neg.	Neg.	Few scattered conglom. tuber. in one node, scattered solitary tuber. in two other nodes.	.....
111	Neg.	Neg.	Neg.	Neg.	Neg.	Few scattered conglom. tuber.	Neg.	One conglom. tuber. in one other two nodes neg.	Non-tuber. haemo area—neg.
107	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	.....
53	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	.....
52	Neg.	Neg.	Neg.	Neg.	No tissue.	Scattered conglom. tubercles.	Neg.	Numerous conglom. tubercles in all three nodes.	.....

In this experiment II, 1927-28, in which the resistance of vaccinated animals and unvaccinated controls to injections of virulent bovine strain are compared, it is shown that whereas the unvaccinated controls exhibited gross lesions in 95 per cent of the animals the vaccinated animals showed *macroscopic* lesions in only 20 per cent and these lesions were of an unprogressive nature.

A study of the records, with respect to the microscopic examinations of the lymph glands of the vaccinated animals, shows that microscopic tuberculous lesions, not evident macroscopically, are to be found in certain of the glands, principally the mediastinal and bronchial glands.

These microscopic lesions were found in 19 out of the series of 25 animals, without the presence of gross lesions in the above noted glands, with the exception of animals Nos. 140, 142, and 119 where small gross lesions were present. (Omitting No. 144 which showed extensive gross lesions in many glands). The other 5 animals, Nos. 133, 118, 114, 107 and 53 were negative both grossly and microscopically.

These lesions may be described in most cases as a few scattered tubercles, a few conglomerate tubercles, or a few scattered conglomerate and solitary tubercles. In the case of the mediastinal glands these references are given for findings in three nodes, meaning representative glands from anterior, middle and posterior mediastinal groups. These microscopic lesions differ markedly from the extensive lesions found in the same glands of the control animals, being practically devoid of caseation and in many cases encapsulated by a fibrous tissue reaction. These findings in the vaccinated animals suggest infection of these animals with *B. tuberculosis* (Vallée) but indicate a marked resistance as contrasted with the extensive lesions in the controls. These animals were examined approximately 3½ months after inoculation.

Some observers may contend that in respect to these microscopic lesions, organisms may be retained in these glands, and be at a later period a source from which may occur a more widely spread and active tuberculosis.

In comparison with results herein reported, may be cited figures given in our report of 1927 in which we stated that ten animals, vaccinated with B.C.G. during 1925 and inoculated intravenously in February 1926 with a virulent strain of tubercle bacilli, when killed in September 1927 (seven months after inoculation) showed six animals free from tuberculosis macroscopically, one doubtful, two with glandular lesions and one with heavy pleuritic, glandular and pulmonary involvement Further, we stated that when triturated glands from these ten animals were inoculated into a series of sixty-three guinea pigs evidence was obtained which proved that nine cattle were still carrying virulent tubercle bacilli while one was apparently free from these organisms.

Experiment No. III. 1927-28.

The Z Group were placed in contact with a tuberculous herd. This group was reduced from fifty to forty-five by the death of five of the controls—one of tuberculosis and four of disease other than tuberculosis, three of which, however, showed tuberculous lesions. Half of this group was sent to contact between September 1 and 5, the others following between September 28 and

October 1, so the whole group was in contact with tuberculous animals (and was fed on milk from a tuberculous source) for a period of at least eight months. The animals were carefully fed with a view to the maintenance of normal growth and health.

TABLE XXII

CONTROLS IN CONTACT WITH TUBERCULOUS ANIMALS

Tag	Date of birth	Date of death or slaughter	P. M. findings
58	Sept. 7	15–6–28	Caseous submaxiliary, large caseo-calcareous, pharyngeal and mediastinal caseo-calcareous.
		Died	
67	Aug. 9	16–6–28	Lungs, caseous spots.
69	Aug. 3	15–6–28	Lungs, bronchial and mediastinal caseo-calcareous.
70	Aug. 17	15–6–28	No macroscopic evidence of tuberculosis.
71	July 30	15–6–28	Lungs caseous (two lesions)
72	July 30	15–6–28	Pharyngeal caseous calcified; lungs two tubercles, large; bronchial caseous calcified; mediastinal caseous calcified.
73	July 27	14–6–28	No macroscopic evidence of tuberculosis.
75	July 27	15–6–28	Pharyngeal slight; lungs caseous; bronchial caseous calcified; mediastinal all affected.
76	July 27	15–6–28	Pharyngeal slight; lungs large caseous; bronchial caseous calcified; marked prescapular slight.
77	July 27	Died 7–5–28	Generalized tuberculosis.
96	July 26	15–6–28	No macroscopic evidence of tuberculosis.
98	July 12	15–6–28	Pharyngeal large caseous calcified. Lungs tubercles in right. Mediastinal middle caseous calcified.
99A	July 11	14–6–28	No macroscopic evidence of tuberculosis.
99B	July 11	14–6–28	Lungs, extensive caseous. Mediastinal post, calcified. Mesenteric slight.
127	Sept. 1	15–6–28	Submaxiliary caseous calcified, Pharyngeal caseous calcified; lungs, bronchial, and mediastinal caseous calcified.
128	Aug. 15	14–6–28	No macroscopic evidence of tuberculosis.
146	Aug. 22	15–6–28	Pharyngeal caseous calcified.
148	Aug. 22	15–6–28	Bronchial calcified.
156	Aug. 15	Died 16–12–27	No macroscopic evidence of tuberculosis.
162	Aug. 15	15–6–28	No macroscopic evidence of tuberculosis.
166	May 7	Died 16–12–27	Lungs caseous.
168	Aug. 15	15–6–28	No macroscopic evidence of tuberculosis.
177	June 14	15–6–28	Pharyngeal caseous calcified extensive. Lungs caseous. Bronchial caseo-calcareous. Mediastinal caseous calcified. Prescapular slight.
179	June 20	14–6–28	Submaxiliary slight caseous spots. Pharyngeal slight. Bronchial slight. Mediastinal ant. caseous calcified. Post. normal.
182	Aug. 25	Died 2–1–28	Caseous pneumonia

Five of the twenty-five unvaccinated control animals in Group Z died before the date of slaughter; all but seven showed macroscopic tuberculous lesions, of a caseous spreading nature in most cases but not in all. In a considerable number of animals there were organic lesions. Seventy-two per cent were tuberculous and 28 per cent free.

TABLE XXIII

VACCINATED IN CONTACT WITH TUBERCULOUS ANIMALS

Tag	Date of birth	Date of vaccination	Date placed in contact with T. B. animals	Slaughtered	P. M. findings
45	23–6–27	24–6–27	Sept. 5	14–6–28	No evidence of T. B.
60	5–8–27	6–8–27	Sept. 5	15–6–28	No evidence of T. B.
63	8–8–27	10–8–27	Sept. 5	14–6–28	Pharyngeal and one bronchial small calcareous area.
65	28–8–27	30–8–27	Sept. 5	14–6–28	Mesenteric caseo-calcareous nodule.
131	20–5–27	23–5–27	Sept. 5	14–6–28	No evidence of T. B.
141	8–6–27	9–6–27	Sept. 28	15–6–28	Lungs one pin head nodule Bronchial caseous. Mediastinal caseo-calcareous.
147	12–6–27	13–6–27	Sept. 28	15–6–28	No evidence of T. B.
149	13–6–27	14–6–27	Sept. 28	14–6–28	No evidence of T. B.
150	15–6–27	17–6–27	Sept. 28	15–6–28	No evidence of T. B.
180	17–6–27	17–6–27	Sept. 5	14–6–28	No evidence of T. B.
181	17–6–27	17–6–27	Sept. 28	15–6–28	No evidence of T. B.
184	29–8–27	30–8–27	Sept. 5	15–6–28	No evidence of T. B.
185	23–6–27	25–6–27	Sept. 5	15–6–28	No evidence of T. B.
186	29–5–27	3–5–27	Sept. 5	14–6–28	One mesenteric caseo-cal.
187	26–6–27	28–6–27	Sept. 28	14–6–28	Pharyngeal slight caseo-calcareous spots.
188	27–6–27	28–6–27	Sept. 28	15–6–28	Pharyngeal caseous lungs small nodule.
189	27–6–27	28–6–27	Sept. 28	14–6–28	Mediastinal small spot calcareous.
190	24–6–27	25–6–27	Sept. 28	15–6–28	No evidence of T. B.
192	2–7–27	3–7–27	Sept. 28	15–6–28	No evidence of T. B.
193	3–7–27	3–7–27	Sept. 28	15–6–28	No evidence of T. B.
195	5–7–27	6–7–27	Sept. 28	15–6–28	No evidence of T. B.
197	10–8–27	11–8–27	Sept. 5	14–6–28	No evidence of T. B.
198	11–8–27	14–8–27	Sept. 5	14–6–28	Lungs small nodule submaxiliary. Pharyngeal and mediastinal calcareous.
199	16–7–27	16–7–27	Sept. 28	14–6–28	No evidence of T. B.
200	23–6–27	27–6–27	Sept. 5	15–6–28	No evidence of T. B.

Eight of these 25 vaccinated animals in Group Z (32 per cent) showed lesions, but they were small and non-progressive in nature, while 68 per cent were macroscopically free from evidence of infection. This was confirmed by microscopic examination.

TABLE XXIV.

MICROSCOPIC EXAMINATION OF THE LYMPH GLANDS OF CONTROLS IN EXPERIMENT III.

Tag	Retroph.	Submax.	Portal	Mesent.	Bronch.	Mediast.	Prescap.	Cervical	Dewlap.*	Lung
179	Neg.	Neg.	Neg.	Neg.	Neg.	Caseous cal. lesions in one gland.	Neg.	Neg.	.....	.....
73	Neg.	Neg.	Neg.	Neg.	Neg.	Eosinophilic infiltrate otherwise neg.	Neg.	Neg.	.....	.....
99(a)	Neg.	Neg.	Neg.	Neg.	Neg.	Old calcified tuber. foci in one node, other nodes neg.	Neg.	Neg.	caseo-cal. lesion.	.....
99(b)	Neg.	Neg.	Neg.	Neg.	Neg. Marked pigment deposit	Neg.	Areas of eosinophilic infiltration otherwise neg.	Neg.	.....	.....
128	Neg.	Neg.	Neg.	Neg.	Neg.	Eosinophilic infiltration neg.	Neg.	Neg.	.....	.....
76	Neg.	Neg.	Neg. Pigment Deposit	Neg. Pigment deposit.	Calcified tuber. lesion.	Neg.	Neg.	Neg.	.....	Sub-acute inflam. peribronch. lymph node. neg.
96	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Fatty tissue only.	.....	.....
168	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	.....	.....

* *Point of vaccination.*

TABLE XXIV, CONTINUED

MICROSCOPIC EXAMINATION OF THE LYMPH GLANDS OF CONTROLS IN EXPERIMENT III.

Tag	Retroph.	Submax.	Portal	Mesent.	Bronch.	Mediast.	Prescap.	Cervical	Dewlap.*	Lung
98	Extensive tuberculous involvem't c̄ calcification.	Neg.	Neg.	Neg.	Neg.	Extensive tuber. lesion of one node with calcification.	Neg.	Neg.	.....	Broncho-pneumonia with marked eosinophilia œdemia.
69	Neg.	Neg.	Neg.	Neg.	Neg.	Extensive tuber. lesion of one nod c̄ calcification.	Periglandular eosinophilic infiltration neg.	Neg.	.....	Sub-acute broncho-pneumonia lesion neg.
177	Extensive fibroid tuber. with calcificat'n	Neg.	Neg.	Neg.	Marked eosinophilic infiltration negative.	Caseo-cal. lesions, one node peri-glandular, eosinophilia one node—3rd node neg.	Neg.	Neg.	.....	.....
148	Neg.	Neg.	Neg.	Neg.	Caseo-cal. (or cervical)	Eosinophilic periglandular infiltration, one node neg.	Neg.	Neg. (see bronch)	.....	.....
75	Neg.	Neg.	Neg.	Neg.	Extensive caseo-cal. lesions.	Extensive caseo-cal. lesions in two glands.	Neg.	Neg.	.....	Extensive caseo-cal. mass in peribronch. lymph node.

* *Point of vaccination.*

TABLE XXIV, CONTINUED

MICROSCOPIC EXAMINATION OF THE LYMPH GLANDS OF CONTROLS IN EXPERIMENT III.

Tag	Retroph.	Submax.	Portal	Mesent.	Bronch.	Mediast.	Prescap.	Cervical	Dewlap.*	Lung
71	Neg.	Neg.	.....	.....	Extensive conglom. tuber. with slight calcification.	Neg.	Neg.	Neg.	caseo-cal. mass.	.....
72	.....	.....	Few small tuber. foci.	Extensive caseo-cal. lesions.	Extensive caseo-cal. lesions.	Extensive caseo-cal. lesions in all three glands.	Neg.	Neg.	Extensive caseo-cal. lesion.	.....
58	Extensive caseo-cal. lesions.	Extensive caseo-cal. lesions.	Neg.	Neg.	Neg.	Extensive caseo-cal. lesion in one gland.	Neg.	Neg.	.....	.....
146	Scattered conglom. tubercles.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	.....	.....
70	Neg.	Neg.	Neg.	Neg.	Neg.	Periglandular eosinophilia of one node. Neg.	Neg.	Neg.	.....	.....
127	No lesions in material sectioned. Neg.	No lesions in material sectioned. Neg.	Neg.	Neg.	Extensive caseo-cal. lesion.	Extensive caseo-cal. lesions in two glands.	Neg.	Neg.	.....	.....
162	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	.....	.....

* *Point of vaccination.*

TABLE XXV.

MICROSCOPIC EXAMINATION OF THE LYMPH GLANDS OF VACCINATED ANIMALS IN EXPERIMENT III.

Tag	Retroph.	Submax.	Portal	Mesent.	Bronch.	Mediast.	Prescap.	Cervical	Dewlap.*	Lung
197	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	.....	.....
187	Caseo-cal. lesions.	Area of conglom. tubercles.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	.....	.....
65	Neg.	Neg.	Neg.	Large caseo-cal. focus.	Neg.	Neg.	Neg.	Neg.	.....	.....
45	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	.....	.....
189	Neg.	Neg.	Neg.	Neg.	Neg.	One gland shows ext'sive tuber. f'm'tion with areas of calcification. Other two glands show numerous eosinophiles surrounding capsules but no lesion in glands.	Neg.	Fatty tissue only.	Chronic inflam. fibrous mass.	.....
149	Neg.	Neg.	Neg.	Neg.	Eosinophilic infiltration small tuber. lesion.	One gland shows marked eosinophilic infiltration, otherwise neg.	Neg.	Neg.	Thoracic inflam. lesion with calcification.	.....
131	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Chronic inflam. fibr. mass.	.....

* *Point of vaccination.*

TABLE XXV, CONTINUED

MICROSCOPIC EXAMINATION OF THE LYMPH GLANDS OF VACCINATED ANIMALS IN EXPERIMENT III.

Tag	Retroph.	Submax.	Portal	Mesent.	Bronch.	Mediast.	Prescap.	Cervical	Dewlap.*	Lung
198	Neg.	Neg.	Neg.	Neg.	Small tuber. focus.	Neg.	Marked eosinophilic infiltration	Neg. (see bronch.)	inflam. mass. c̄ calcification and purulent foci.	Chronic inflam. Pleuritic thickening. Lung—neg.
180	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Chronic inflam. mass.	.....
199	Neg.	Neg.	Neg.	Neg.	Neg.	Some eosinophilia neg.	Neg.	Neg.	Chronic inflam. tissue.	.....
186	Neg.	Neg.	Neg.	Small subacute inflam. area with tubercles.	Acute lymphodenites neg.	Marked periglandular eosinophilic infiltration neg.	Neg.	Eosinophilia neg.	Chronic inflam. mass with tuber. and calcification.	.....
150	Neg.	Neg.	Neg.	Neg.	.....	Neg.	Neg.	Neg.	Fibro-fatty tissue c̄ areas of chronic inflam. cell infil.	.....
147	Neg.	Neg.	Neg.	Neg.	Marked eosinophilic infiltration—neg.	Marked eosinophilic infiltration periglandular neg.	Neg.	Neg. Eosinophilic infil. (or bronch.)	.....	.....
184	Neg.	Neg.	Neg.	Neg.	Neg.	.....	Neg.	Neg.	.....	.....
63	Tuber. lesion.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	.....	.....

* *Point of vaccination.*

TABLE XXV, CONTINUED

MICROSCOPIC EXAMINATION OF THE LYMPH GLANDS OF VACCINATED ANIMALS IN EXPERIMENT III.

Tag	Retroph.	Submax.	Portal	Mesent.	Bronch.	Mediast.	Prescap.	Cervical	Dewlap.*	Lung
188	Extensive conglom. tubercles slight caseation.	Neg.	Neg.	Neg.	Neg.	Periglandular eosinophilic infiltration neg.	Neg.	Neg.	.....	Slight chronic inflam. lesion, not definitely tubercular.
190	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Chronic inflam. lesion with caseous areas.	.....
185	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	.....	.....
192	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	.....	.....
181	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	.....	.....
141	Neg.	Neg.	Neg.	Neg.	One solitary tuberculous lesion—small	Extensive caseo-cal. lesion in one gland.	Neg.	Neg.	.....	Minute chronic inflam. lesion not definitely tuber.
200	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	.....	.....
195	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	.....	.....
60	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	.....	.....
193	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	.....	.....

* *Point of vaccination.*

Discussion

Rabies is one of the outstanding infectious diseases of animals, which, for a considerable period of time, has been successfully controlled by the destruction of the animals infected, coupled with quarantine and other methods. The control of *B. tuberculosis* infection has been assisted considerably by the method of the establishment of tuberculosis-free herds in certain districts, an inverted method of quarantine, based upon the tuberculin reaction, and supplemented by the destruction of the infected animals so determined. In other conditions the methods employed have been those of quarantine, sometimes coupled with immunization, largely through the agency of vaccines.

In consideration of some of these conditions it must be admitted that considerable success has been attained *both in animals and man* by the use of methods of immunization, which give not absolute but only relative immunity.

There is, however, very little for comparison between the acute condition of rabies, transmitted usually by direct contact, and the insidious condition of tuberculosis widespread as it is to-day amongst humans and domestic animals, and now further complicated by the recently put forward possibility of transmission through the agency of a filterable form of the organism.

*In all methods of quarantine whereby the infected are more or less effectively separated from the uninfected, one of the great fallacies is the continued infection of the uninfected through the agency of unrecognized cases of infection, or by infection through contact, direct or indirect, with carriers of the cause of the infection who, themselves, may show no evidence of infection. Such, in fact, has been a disadvantage with respect to the methods based on the tuberculin reaction for the control of *B. tuberculosis* infection in bovines; and this has been further complicated by the fact that, like all biological reactions, the tuberculin reaction is subject to a certain percentage of error (small though it is) and the further fairly well established fact that tuberculosis-free herds are more susceptible to tuberculous infection than are, generally speaking, the ordinary run of animals which have survived, and which have in some cases possibly thrown off infection. Instances of this have come under observation due to a break in the quarantine as a result of the introduction of animals from outside sources which have reacted negatively to the tuberculin test, although it must be admitted that there may have been in some cases, another explanation—as for instance the introduction simply of an infected animal, or that the infection may have been acquired as the result of contact with animals of another species subject to infection with *B. tuberculosis* of bovine or possibly other strain. The contention for purpose of argument that immunized animals might be a menace by virtue of their being carriers of a virulent strain, or by virtue of the danger of the strain by which they were immunized becoming virulent and so a menace to unvaccinated animals, does not dispose of the question even if it were proven true, for the reason that unprotected and infected animals, and possibly animals of another species, are capable of spreading disease, and even animals which have been negative to the tuberculin reaction have been incriminated as the origin of infection.

* *References 12, 13, 14, 15, 16.*

It follows, we think, from all this that the resistant animal would appear to be the ultimate basis upon which methods of prevention (in man and animals) will eventually rest their foundations, either by the establishment of a resistant strain (in the case of animals) or other means, and certainly inseparable from all subsidiary methods for the maintenance of resistance, such as food, etc. These remarks are not prompted by any spirit of criticism, nor with the least purpose of unconsidered interference with methods which despite their imperfections, have at least accomplished something; but for the purpose of pointing out some of the reasons why a method which aims at the production of resistance is worthy of investigation. The refutation or establishment of the claims for such methods must, however, stand upon the experimental results obtained and their intelligent interpretation.

Summary of Results

As reported to the National Research Council from time to time, our results, within the limits of the experiments, have given percentages of protection, as estimated by the absence of macroscopic or microscopic lesions in vaccinated animals exposed to the possibility of infection by contact, varying from 22 per cent in our second experiment (Group V, reported in May, 1927, as 44 per cent macroscopically) to 100 per cent in our third experiment, in which, however, the conditions of experiment were different in that the animals were not placed in contact for a period of two months following vaccination, during which time they were fed on pasteurized milk. (Group VI, reported in May, 1927, on macroscopic grounds and now confirmed microscopically).

The average percentage in four experiments: Group I, reported June, 1926, and Group V, as above, *under immediate contact conditions* numbering 25 animals, and Group VI, as above, and Group Z reported December, 1928, *under delayed contact conditions* numbering 41 animals—in all *66 vaccinated animals*, over periods varying from 4 to 11 months—is 66 per cent, confirmed by microscopic examination except in the case of Group I, numbering 16 animals. On the other hand, unvaccinated controls used in these experiments numbering 43 showed only 8 per cent free from tuberculous lesions, that is to say, 92 per cent were tuberculous. In the last two contact experiments involving 41 vaccinated animals over periods varying from 8 to 10 months, 80 per cent were grossly and microscopically free from tuberculosis, while unvaccinated controls numbering 32 animals showed only 14 per cent free from tuberculous lesions.

66 Vaccinated animals exhibit 66 per cent free from tuberculous infection.
41 Vaccinated animals exhibit 80 per cent free from tuberculous infection.
43 Unvaccinated controls show 8 per cent free from tuberculous infection.
32 Unvaccinated controls show 14 per cent free from tuberculous infection.

In the inoculation experiments, with *B. tuberculosis* (Vallée), involving 35 vaccinated animals (Group II & Y) and 34 unvaccinated controls (Group II & Y), there is marked evidence of resistance in the vaccinated animals.

35 Vaccinated animals exhibit marked resistance to intravenous inoculation with virulent bacilli.

34 Unvaccinated controls show less resistance to intravenous inoculation with virulent bacilli.

All these results, where controls and vaccinated animals have been subjected to the same conditions, demonstrate resistance, and show, even in the majority of infected vaccinated animals, a marked difference in the lesions obtained when compared with the controls.

Our two experiments with 20 animals indicate complete absence of lesions macroscopical or microscopical following simple vaccination with B.C.G. after 7 months in the first experiment numbering 10 animals, and one year in the second experiment numbering 10 animals.

20 Animals do not show lesions following vaccination with B.C.G.

Conclusions

(1) Vaccination with B.C.G. produces resistance in bovines to tuberculous infection.

(2) B.C.G. is non-virulent for bovines.

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STUDIES ON THE NATURE OF RUST RESISTANCE IN WHEAT[1]
IV. PHENOLIC COMPOUNDS OF THE WHEAT PLANT

By R. Newton[2] and J. A. Anderson[3]

Abstract

It is suggested that rust resistance in wheat may be due to the liberation of phenols in the host-cell upon the entrance of the fungus. A tentative method has been developed for determining phenolic compounds in wheat press-juice, including a critical study of the conditions for clarifying the juice with tungstic acid. The content of phenolic substances in wheat varieties has been found to bear some relation to rust resistance. Yellow pigments of the flavone type appear to be the main phenolic compounds present, and these are being investigated.

Various hypotheses which have been put forward to explain resistance or susceptibility to disease in plants, and the grounds on which these are based, have been discussed in an earlier paper by Newton, Lehmann and Clarke (21). In the same paper a broad program of studies on the nature of resistance and susceptibility to stem rust in wheat was outlined. This included an investigation of the host-cell contents in a search for substances which might be toxic to the fungus. For reasons which are set forth below, the phenolic substances have been selected for first study, and this paper reports the initial stages of an investigation of this class of compounds in the wheat plant.

Plant Substances Toxic to Disease Organisms

A review of the literature on disease resistance offers certain indications of the class of compounds which may play a part in resistance as toxic substances. Cook and Taubenhaus (4, 5) found cases in which disease resistance was apparently correlated with the rate of tannin formation in the host cells. Working with sixty-six organisms, they also found that tannic acid was more toxic than a variety of other vegetable acids, and that in general it was more toxic to parasitic than to saprophytic fungi. Cook and Wilson (6), working with members of the genus *Endothia* in culture media, found that in most cases growth was retarded by 0.8 per cent of tannin. *Endothia parasitica*, however, was able to use tannin as a food. In general, they claim that tannin inhibits fungal growth and that the tannin content of the cells is one factor in the resistance of certain plants to attack by fungous diseases.

Graves (12) investigated the cause of persistent development of vigorous basal shoots from the roots of chestnut trees which had been killed by blight.

[1] *Manuscript received Jan. 31, 1929.*
Contribution from the laboratories of the University of Alberta, Edmonton, Alberta, Canada, with financial assistance from the National Research Council of Canada. These studies form part of a co-operative attack on the problem of cereal rust in Canada, carried on jointly by the National Research Council, the Federal Department of Agriculture and the Universities of Alberta, Manitoba and Saskatchewan. The present paper is the second of a series, the first of which, consisting of Parts I, II and III, appeared in the Canadian Journal of Research, 1:5-35, May, 1929.
[2] *Professor of Field Crops and Plant Biochemistry, University of Alberta.*
[3] *Post-graduate student, University of Alberta.*

The parasite was found to grow more slowly in the roots than in the stems and it was suggested that this is because the roots contain twice as much tannin as the stems.

Hawley, Fleck and Richards (15) found a high correlation between the keeping quality of wood, and the toxicity of water extracts of the wood to fungi. The toxic properties of the extracts are attributed to tannins and water-soluble colouring matter of unknown composition.

Walker (29) investigated smudge in onions. Coloured varieties were found to be resistant as a class whereas colourless varieties were susceptible. The pigments are confined to the dry outer scales. The author offers evidence showing that the chief factor causing resistance to smudge is apparently a substance closely associated, if not identical, with these pigments. Their functioning, according to Walker and Lindengren (31), depends on the ready diffusibility of these pigments out of the dead tissue into the soil water, where they inactivate the fungus before attack. The press-juice of the inner scales of the onion was also toxic to fungi, but in this respect resistant and susceptible varieties were alike. The authors therefore conclude that the cell sap is not an initial factor in resistance but may be important in limiting growth after the fungus has entered. In later work with neck rot of onions, Walker (30) offers the same explanation of resistance.

Tins (27) suggests that certain varieties of cabbage owe their resistance to cabbage yellows to a greater content of sulphur-containing compounds, but offers no experimental evidence on this point. Reynolds (23) attributes resistance to wilt in certain varieties of flax to a higher content of the glucoside linamarin, which can be split by the enzyme phaseo-lutinase to produce free hydrocyanic acid.

The Phenol Hypothesis

Of the substances mentioned above, it would seem that those most likely to occur in wheat are the water-soluble pigments, related compounds, and tannins. A consideration of the structural formulas of these two classes of compounds shows that they are related in that the molecules of both contain various simple phenols. This suggests that their toxicity may be due to their phenolic constituents, since it is well known that many of the simple phenols have antiseptic and germicidal properties. With these considerations in mind a tentative hypothesis which would form the basis of an experimental investigation was drawn up as follows: resistance to stem rust in wheat is due to phenolic compounds set free in the cell on the entrance of the fungus; these kill the cell and inhibit the growth of the parasite.

The possible mechanism of such a reaction may be elaborated along various lines. If the toxin acts upon the host-cell and fungus by denaturation or coagulation of the protoplasmic proteins, it evidently cannot exist free in the cell under normal conditions. It may, however, be present in harmless combinations, as in pigments, tannins, and glucosides. These may be broken down by enzymes secreted by the fungus, thus setting free the toxic constituents.

To account for the specificity of the wide variety of rust forms, we may invoke the well-known specificity and variety of enzymes which occur, and the possibility of certain ones being peculiar to certain rust forms. Again, the phenolic compounds in the various wheat varieties may differ both in their abundance and in their resistance to enzymic decomposition, thus offering further possible explanations of differential rust reactions.

Another possibility is suggested by the work of Klotz (17). This author found that the bark of the sour orange tree had a much greater inhibitory action on certain enzymes found in the dry powdered mycelium of the organism causing gummosis, than had the bark of the lemon tree. The sour orange is resistant to this trouble, while the lemon is susceptible. Enzyme inactivators may occur similarly in certain wheat varieties, and retard or prevent the decomposition of phenolic compounds, thus apparently leaving such varieties open to successful invasion by the rust fungus. Conversely, enzyme activators may be postulated as a part of the mechanism.

If the relative abundance or the natural resistance to enzymes of the particular phenolic compounds in a given wheat variety, or the presence in such variety of an enzyme inactivator, or again the relative quantity or activity of the fungal secretion, should lead to only a moderate amount of decomposition and a resultant low concentration of the active constituent in the cell, there may follow, not inhibition, but stimulation of both host and parasite. In the first paper of this series (21) it was shown that the first result of adding certain phenols to a fungous culture in minimum effective concentration was a stimulation of growth, which increased with concentration up to an optimum and then decreased to the maximum limit of tolerance. It may well be that the observed stimulation of the invaded cells of rust-susceptible wheat varieties is brought about by moderate decomposition of phenolic substances, that the death of the cells of resistant varieties in similar circumstances is owing to excessive release of the active constituents, and that the occasional stimulation of resistant cells more remote from the original point of infection is in turn caused by the outward diffusion, with gradual dilution, of the free compounds.

Small differences in the structure and composition of phenolic compounds have been shown to affect their antiseptic and germicidal efficiency considerably. Johnson and Lane (16) found that the incorporation of an alkyl group in the aromatic nucleus of resorcinol increased its antiseptic properties, and that the increase was a function of the size of the group introduced. Tilley and Schaffer (26) found that the same held true for the germicidal activity of phenol. Similarly, in a survey of phenolic fungicides, Gray (13) reported that cresols were more effective than phenol. It appears therefore that either small differences in the original composition of phenolic compounds occurring in different wheat varieties, or in the way in which they are split by the enzymes of specific rust forms, may have a marked effect on their reactions to such forms.

Cooper and Mason (7) classified germicides as "chemical" or "physico-chemical", their actions depending respectively upon chemical reactivity with protoplasmic constituents and denaturation or precipitation of the cell proteins. They found that *B. fluorescens non-liquefaciens* was selectively attacked by physico-chemical germicides such as phenol, while *B. coli* was less sensitive to them, but more sensitive to chemical germicides such as the quinones. Substitution of various groups in the aromatic nucleus affected the germicidal power on these two bacteria unequally, with the result that the selective action might be obscured or even reversed. These findings provide further evidence of the importance of small differences in the composition of the active substance, and also suggest the possibility that a given phenolic compound may differ in its toxicity to the different forms of rust.

It seems almost too much to hope that the relatively simple suggestions put forward above will serve to explain all the complications of rust reactions in wheat. However, a more specific working hypothesis than those based on cytological investigations was absolutely essential in order to narrow the field, and since none more plausible suggested itself, the phenol hypothesis was tentatively adopted.

To test this hypothesis, the following steps suggested themselves: (1) to make a qualitative investigation of the phenolic compounds occurring in wheat plants; (2) to follow this with an attempt to develop quantitative methods for comparing the abundance of individual compounds in different wheat varieties; (3) to investigate the physiological activity of the compounds discovered towards the cell contents of different wheat varieties; (4) to determine the change in physiological activity resulting from contact with the secretions of various rust forms.

Analytical Studies

We have so far been unable to find any definite information in the literature on the phenolic compounds occurring in wheat plants. Tannins are almost universally present in plants. Shibata, Nagai and Kishida (25) find that flavone derivatives are very widely distributed, and they may therefore be expected to occur in wheat. Collison (3), working on the toxicity of extracts of plant residues to the growth of seedlings, obtained qualitative tests for vanillin in extracts from wheat straw, and tests for vanillin and salicylic acid in extracts of alfalfa hay. (It is interesting to note that he suggests that some of these rare substances with antiseptic properties may play a part in the prevention and limitation of fungal attack.)

The program outlined above for testing the phenol hypothesis is clearly a long and difficult one to carry through. Ignorance of the phenolic compounds present in wheat, and the probability of such compounds occurring only in very small amounts, complicate the investigation. It was therefore decided to make, as a preliminary step, a general quantitative survey of the phenol content of the eight wheat varieties of graded resistance to rust, which have been used throughout our rust investigations, and if evidence favouring the hypothesis was secured, to make the more detailed investigation later.

As analytical material, we used mainly the press-juice of young wheat plants, grown in field plots under normal conditions. The juice was extracted by the method of Newton, Brown and Martin (20). Some preliminary work with the air-dried, ground tissues of the same kind of plants is also reported.

Since we have no knowledge of the phenols which occur in wheat, or of the substances present which may interfere with the determinations, the term "phenol" as used in the following pages must be interpreted in conjunction with the methods of extraction and determination used.

Methods for the Quantitative Determination of Phenols

Since quantitative determinations of phenols have been made on such complex physiological fluids as blood (1, 22) and urine (28), there appeared to be no *a priori* reason why one of the methods should not be adapted to the determination of phenols in the press-juice of wheat leaves. Gibbs (10) has reviewed the literature on phenol tests, reporting more than forty tests varying in the character of reagent. The older volumetric methods have been largely discarded in favour of colorimetric methods. The colour reagent of Folin and Denis (8) has been used almost exclusively for physiological fluids. This reagent is not specific for phenols. Gortner and Holm (11) have pointed out that it reacts with a number of protein degradation products, and Levine (18) finds that it gives a colour with a great variety of compounds and concludes that, in general, it reacts with all sorts of compounds having more or less reducing properties.

Since the Folin-Denis reagent is not specific for phenols as a class the problem of determining phenols by its use is largely one of getting rid of interfering substances. In the recent work on blood and urine, the most important interfering substances have been precipitated by various reagents. In earlier work on urine, reviewed by Tisdall (28), several methods were developed for the separation of volatile phenols by steam distillation. Extraction of the phenols from acid solution with ether, followed by extraction from ether with dilute sodium hydroxide solution, has been used by Tisdall (28) and Hanke and Koessler (14). Both these methods appeared applicable to the problem in hand.

Colorimetric Method Adopted

After reviewing the literature on the use of the Folin-Denis phenol reagent, and experimenting with several methods, the technique of Rakestraw (22) was adopted. The reagent was made up according to the improved method due to Wu (32). A fresh portion of the reagent was diluted with three parts of water before using, and 0.5 cc. of this solution was found to be sufficient for the development of maximum colour in 10 cc. of the phenol solutions. After adding the reagent to 10 cc. of the solution in a test tube and mixing, 2 cc. of 20 per cent sodium carbonate solution was added. The solution was then allowed to stand until the excess reagent had been destroyed by the carbonate. Rakestraw found ½ min. sufficient, but Chapin (2) found that 20 min. was required and he adopted a standard time of 30 min. Under our

conditions Chapin's technique was found to be necessary. After allowing the mixture to stand 30 min., 1 cc. of 5 per cent sodium cyanide solution was added and the test tube was stood in boiling water for 1½ min., when it was transferred to cold running water for 3 min. The color developed was found to remain constant for at least two hours, but colorimetric readings were generally made immediately. Comparisons were made in a Leitz-Buerker colorimeter with a standard solution of resorcinol treated by the same method. Resorcinol was adopted as a standard by Benedict and Theis (1) since it can be weighed out directly.

The quantitative accuracy of the method was tested on resorcinol solutions and on an extract from the press-juice of wheat. A series of solutions of different concentrations was made up. One of these was selected as a standard and the concentrations of the others were determined against it in the colorimeter. The results, expressed in both cases as resorcinol equivalent in mg. per litre, are reported in Table I. They showed a satisfactory accuracy for the present investigation, and the method was adopted for the work reported in the following pages.

TABLE I.

ACCURACY OF DETERMINATION WITH THE FOLIN-DENIS REAGENT

Extract		Resorcinol			
Determined	Present	Determined	Present	Determined	Present
3.9	3.8	6.9	7.0	6.3	6.3
3.5	3.4	5.4	5.3	5.5	5.6
3.0	Standard	4.1	4.2	4.8	4.9
2.9	2.7	3.5	Standard	3.5	Standard
2.3	2.3	3.3	3.2	2.9	2.8
1.9	1.9	2.9	2.8	2.2	2.1
1.6	1.5	2.2	2.1	1.8	1.8

ATTEMPT TO DETERMINE VOLATILE PHENOLS OF PRESS-JUICE BY DIRECT DISTILLATION

A 350-cc. portion of press-juice of Khapli wheat plants was steam distilled, and the distillate collected in 100-cc. portions. Determinations of the amount of phenol in each portion of distillate were made with the Folin-Denis reagent. The results are reported in Table II. These results show that it would be impracticable to attempt quantitative comparisons of the amounts of volatile phenols in different wheat varieties by this method, since there is no indication of an endpoint at which the distillation might reasonably be stopped.

TABLE II.

AMOUNT OF PHENOL IN SUCCESSIVE 100-CC. PORTIONS OF THE STEAM DISTILLATE OF PRESS-JUICE

Portion number	Resorcinol equiv.	Portion number	Resorcinol equiv.	Portion number	Resorcinol equiv.
1	6.1	9	2.4	17	1.8
2	3.1	10	1.9	18	1.7
3	3.0	11	2.1	19	1.9
4	2.7	12	2.2	20	1.7
5	2.3	13	2.7	21	1.7
6	2.2	14	1.7	25	2.0
7	2.3	15	1.7	30	1.8
8	2.5	16	1.9		

DETERMINATION OF PHENOLS OF PRESS-JUICE BY ETHER EXTRACTION

As has been previously pointed out, the problem of determining phenols colorimetrically is mainly that of getting rid of interfering substances. Phenols can be extracted from an acidified solution by ether and extracted from ether by alkaline solutions. An attempt was made therefore to use this method for the extraction of phenols from the press-juice of wheat.

A preliminary experiment showed that it would be necessary to precipitate the proteins and chlorophyll from the press-juice before extracting with ether. This was effected with the tungstic acid reagent of Folin and Wu (9), which has been widely used for clearing blood on which determinations of phenols were to be made. The investigation of the optimum conditions for precipitation is reported in a later section of this paper.

Hanke and Koessler (14), working with solutions of pure phenols, found that ten extractions with twice the volume of ether were required to effect quantitative removal. This point was investigated for our conditions. A 10-cc. portion of the filtrate from the tungstic acid precipitation was extracted eleven times with 20 cc. of ether. Each ether extract was in turn extracted with 2.5 cc. of 10 per cent sodium hydroxide solution. These alkaline extracts decreased successively in depth of colour from bright yellow to almost colourless. Each was neutralized with dilute hydrochloric acid, and after being made up to a volume of 25 cc. its phenol content was determined. The results of two such series of determinations made on the press-juice of Khapli plants are reported in Table III.

TABLE III.

EFFICIENCY OF ETHER EXTRACTION OF PRESS-JUICE

Extraction number	Phenols as resorcinol in mg.	
1	1.500	1.250
2	0.408	0.350
3	0.145	0.108
4	0.070	0.055
5	0.043	0.030
6	0.040	0.023
7	0.030	0.015
8	0.020	0.013
9	0.013	0.010
10	0.015	0.008
11	0.010	0.008
2nd 5 extractions as per cent of 1st 5	5.4	3.9
11th extrn. as per cent of 1st 10	0.44	0.43

Since the amount of phenol decreased steadily with each extraction, and since the amount in the 11th extract was less than 0.5 per cent of that in the first ten extracts, ten extractions with twice the volume of ether were adopted as a standard number.

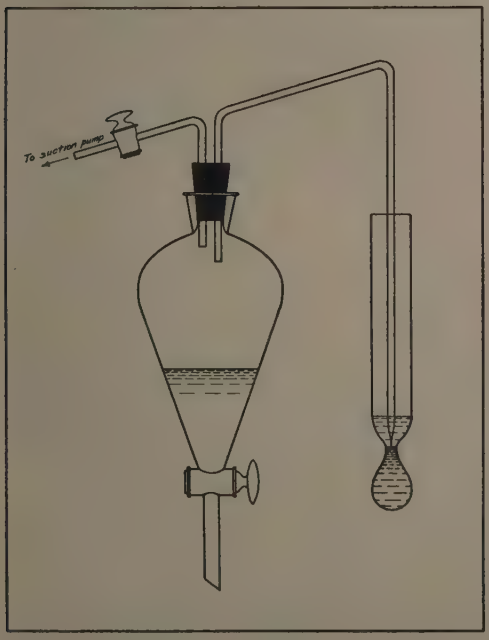

FIGURE 1— *Apparatus used in extraction of phenols from press-juice*

The number of extractions required to remove the phenol from the ether was also investigated. The combined ether extracts, having a volume of 200 cc., were extracted three times with 10 cc. of 10 per cent sodium hydroxide solution and these extracts were combined (Extract 1). The ether was then extracted twice more with 5 cc. of sodium hydroxide solution; these were also combined (Extract 2). It was found that, after neutralising, these solutions would have to be diluted to 10 times their volume in order to prevent the salt concentration interfering with the colorimetric determination. In only one out of three determinations did sufficient colour

develop in Extract 2 for quantitative comparison. In this case the amount of phenol in Extract 2 was 0.22 per cent of that in Extract 1. Three extractions with 10 cc. of 10 per cent sodium hydroxide solution were therefore adopted as a standard number.

A description follows of the method of precipitation and extraction as finally adopted. A 25-cc. portion of the press-juice was pipetted into a 100-cc. centrifuge tube, 5 cc. of 15 per cent sodium tungstate ($Na_2WO_4.2H_2O$) solution was added, followed by 2.5 cc. of 10 per cent sulphuric acid. The solution was shaken, centrifuged at low speed for five minutes, and decanted through a filter. A 10-cc. aliquot of the filtrate was pipetted into an extraction tube with a 10-cc. bulb, shown in Figure 1, and extracted ten times with 20-cc. portions of ether, the ether being drawn off each time into a separatory funnel, also shown in the figure The ether extracts, combined in the separatory funnel, were then extracted three times with 10-cc. portions of 10 per cent sodium hydroxide solution. The bright yellow alkaline extract was made just acid to litmus paper by titrating with dilute hydrochloric acid, and turned to a very pale yellow colour. The solution was then made up to volume in a 250 cc. volumetric flask, and an aliquot was used for determination of phenols with the Folin-Denis reagent.

Both volatile and non-volatile phenols were determined on this solution. A 200-cc. portion was measured out and washed into a 500-cc. distillation flask with 50 cc. of distilled water. When 100 cc. of distillate had collected, 50 cc. of water was added through a separatory funnel set in the stopper of the flask. A further 50 cc. was distilled off, another 50 cc. of water added and a fourth 50-cc. portion distilled. The solution remaining in the distillation flask was washed back into a 250 cc. volumetric flask and made up to volume. The concentration of phenol in the distillate was too low for determination. The phenol in the remainder was determined and the volatile phenols were obtained by difference.

Phenol content of wheats varying in rust resistance

The above method for the determination of phenols was applied in the summer of 1927 and again in the summer of 1928 to a series of eight wheat varieties of graded resistance to rust. The phenol contents found in the press-juice of the young plants are reported in Table IV. The results of duplicate determinations are shown separately in the 1928 series, and considering the empirical nature of the method these exhibit a satisfactory agreement.

TABLE IV.

PHENOL CONTENT OF PRESS-JUICE OF WHEATS.

VARIETIES ARRANGED IN PROBABLE ORDER OF RESISTANCE TO STEM RUST.

Variety	Phenol as resorcinol mg. per 100 gm. juice		
	Total	Non-volatile	Volatile
1927			
Khapli	15.6	12.4	3.2
Vernal	9.1	7.8	1.3
Kanred	5.9	4.3	1.6
Kota	10.7	7.7	3.0
Kubanka	16.0	11.0	5.0
Mindum	11.8	7.8	4.0
Marquis	5.9	5.0	0.9
Little Club	5.5	3.6	1.9
1928			
Khapli	12.7	6.4	6.3
	12.4	6.3	6.1
Vernal	6.4	4.4	2.0
	6.3	4.5	1.8
Kanred	6.3	5.4	0.9
Kota	5.8	3.6	2.2
	6.5	4.0	2.5
Kubanka	12.9	6.7	6.2
	12.0	6.3	5.7
Mindum	8.1	4.0	4.1
	7.9	4.0	3.9
Marquis	5.3	4.6	0.7
	5.4	4.4	1.0
Little Club	5.5	3.3	2.2
	5.5	3.4	2.1

Of the varieties used in these studies, Khapli is highly resistant to all known forms of rust but one, Marquis and Little Club are extremely susceptible, and the other varieties show different degrees of resistance and susceptibility to the various rust forms. The results of the phenol determination show that Khapli contains considerably more phenols than all other varieties but Kubanka; Marquis and Little Club contain least phenols, and the other varieties fall between these two extremes, but not in the order of their resistance to rust. It must of course be borne in mind that differences in the concentration of phenols constitute only one of the possible ways suggested in which these substances may play a part in the rust reactions of different varieties.

Since we have no knowledge of the individual phenols and interfering substances present in the press-juice, the phenol determination is purely empirical. For this reason, too much weight should not be placed on the results obtained. However, we consider them encouraging, and further work on the phenol hypothesis is under way.

Qualitative Analysis

Before undertaking further quantitative studies it seemed advisable to determine the individual phenolic compounds present in the wheat plant. Colour changes during the course of previous work led us to believe that the main phenolic compounds present were yellow pigments, possibly of the flavone type.

A preliminary qualitative analysis of 3200 gm. of air-dried, ground tissues of the young plants has been made. The powder was extracted by boiling with ten times its weight of water under a reflux condenser. The mixture was strained through a cloth and the filtrate was treated with excess lead acetate solution, followed by excess ammonium hydroxide solution. The precipitate was decomposed by suspending in boiling alcohol and passing in hydrogen sulphide. Further separations were carried out by differential solubility in various organic solvents.

The compounds present occur in very small amounts and are difficult to separate in pure form. It is believed that three different pigments have been shown to be present. These have not been obtained in crystalline form, but appear to be the main phenolic compounds in the wheat. Seven other compounds were obtained in crystalline form, but in such small quantities that they have not so far been purified. These compounds do not appear to be phenolic.

An analysis of a much larger amount of wheat tissue is now under way with the object of isolating sufficient quantities of the pigments, in pure form, for the determination of their properties and for their identification.

Investigation of the Precipitation of Proteins from Press-juice of Wheat by Tungstic Acid

In the precipitation of nitrogenous materials by the tungstic-acid reagent of Folin and Wu (9) a certain degree of acidity is necessary. In precipitation from blood the above-named investigators added a solution of 10 per cent sodium tungstate and an equal quantity of 2/3N. sulphuric acid. This gave a slight excess of acid over that required to neutralise the combined alkalinity of the sodium tungstate and the blood serum. Rumsey (24) adapted this method to the precipitation of proteins from cereal extracts. The original technique was not satisfactory owing to the high buffer value of the phosphates and proteins present, which prevented a sufficient increase in the hydrogen-ion concentration. Placing this on a pH basis, he found that optimum precipitation of nitrogenous compounds and clarification of the solution occurred at a pH of 2 or less, which he obtained by adding concentrated sulphuric acid

to the end-point of thymol blue. Merrill (19) investigated the precipitation of protein from diphtheria antitoxin serum and from peptone solutions. With the former, the zone of maximum precipitation occurred at pH 5.0 with a slight increase at pH 2.8. With the latter, the precipitation increased considerably as the pH decreased from 4.0 to 1.0, with a slight reversal of precipitation beyond this, at the acidity of 10 per cent sulphuric acid. It appears therefore that the optimum conditions of acidity for maximum precipitation vary with the nitrogenous compounds present. For this reason an investigation was made of the conditions necessary for the maximum precipitation of nitrogenous compounds from the press-juice of wheat.

Preliminary experiments showed that at a given acidity, precipitation increased with increasing amounts of sodium tungstate, and that with a given amount of sodium tungstate, precipitation increased with increasing acidity, though reaching an approximate maximum at about pH 3 to 2.

Two series of determinations were therefore made in order to determine the amount of tungstate necessary for complete precipitation. Different amounts of 15 per cent sodium tungstate ($Na_2WO_4.2H_2O$) solution, together with an amount of 10 per cent sulphuric acid which would give an approximate pH of 2, were added to 10 cc. of press-juice. After filtering and washing with hot water, nitrogen determinations were made on the precipitate and on the combined filtrate and washings. Hydrogen-ion concentrations were determined potentiometrically on a separate portion of undiluted filtrate. The results are reported in Table V. With certain irregularities, they show a slight increase of precipitation with increasing amounts of sodium tungstate, but it would appear that 2 cc. of 15 per cent sodium tungstate solution is sufficient for the precipitation of the proteins from 10 cc. of press-juice.

TABLE V.

EFFECT OF DIFFERENT AMOUNTS OF SODIUM TUNGSTATE ON THE PRECIPITATION OF PROTEINS 10 cc. of press-juice

15 per cent sodium tungstate	10 per cent H_2SO_4	Acidity of filtrate	Nitrogen in filtrate	Nitrogen in precipitate
cc.	cc.	pH	mg.	mg.
Kanred, grown in the field				
1	0.92	1.69	11.5	44.7
2	1.06	..	10.7	46.3
3	1.20	..	10.2	46.1
4	1.34	..	10.5	45.9
5	1.48	..	10.1	46.0
6	1.62	..	10.8	45.5
7	1.76	2.73	10.6	45.7
Khapli, grown in the greenhouse				
1	0.85	1.46	11.0	20.8
2	1.00	1.64	10.2	21.2
3	1.22	1.55	10.0	21.3
4	1.40	1.55	10.0	21.5
5	1.58	1.49	10.3	21.7
6	1.76	1.38	10.2	22.1

Two series of determinations were also made in order to study the effect of hydrogen-ion concentration on the efficiency of the precipitation. The results are reported in Table VI. They show an increase of precipitation with increasing hydrogen-ion concentration, but this increase is comparatively small after a pH of about 2.0 has been reached.

TABLE VI.

EFFECT OF HYDROGEN-ION CONCENTRATION ON THE PRECIPITATION OF PROTEINS

10 cc. of press-juice plus 2 cc. of 15 per cent sodium tungstate solution.

10 per cent H_2SO_4	Acidity of filtrate	Nitrogen in filtrate	Nitrogen in precipitate
cc.	pH	mg.	mg.
Khapli, grown in the greenhouse.			
0.5	4.0	14.1	24.1
0.7	2.45	13.0	25.1
1.0	1.68	11.7	26.0
1.3	1.30	11.8	25.9
1.7	1.09	11.7	26.2
0.5 conc.	..	11.7	26.0
Kanred, grown in the field.			
0.5	4.60	19.6	30.0
0.7	3.20	15.9	33.4
1.0	2.04	14.9	34.7
1.3	1.50	14.9	34.0
1.7	1.08	14.4	35.2
0.5 conc.	..	14.3	35.0

Since it was important to obtain a concentrated filtrate for the phenol determinations the following method, based on these results, was adopted: Add to ten volumes of press-juice, two volumes of 15 per cent sodium tungstate solution followed by one volume of 10 per cent sulphuric acid. This procedure gives a clear yellow filtrate of about pH 2.0.

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THE EFFECT OF THE ULTRA-VIOLET COMPONENT OF SUNLIGHT ON CERTAIN MARINE ORGANISMS.[1]

By A. Brooker Klugh[2]

Abstract

In order to determine the effect of the ultra-violet and other components of sunlight upon certain marine organisms an apparatus was constructed with three different light filters. Seven marine organisms, varying widely in their habits with reference to light, were then exposed in the apparatus to various degrees of sunlight. Through one filter they received 91 per cent of the total sunlight, comprising both the visible range and the ultra-violet; through another, 82 per cent of the total, including the visible spectrum but no ultra-violet; through the third, 14 per cent of the total radiation, at the short-wave end of the spectrum. A fairly close relation was observed between the depth at which the organisms live and their susceptibility to short-wave radiation. In several cases ultra-violet light was found to be lethal in its effect. (F. E. L.)

Introduction

This research was suggested by the fact that certain marine organisms, especially some species of copepods, remain at considerable depths in the ocean during daylight, and even during bright moonlight, and come to the surface only on the darkest nights, and also by the fact that in some recent experiments, carried on at the Atlantic Biological Station, St. Andrews, N.B., we found that the copepod *Calanus finmarchicus* showed a higher mortality when exposed under a blue filter than under a red or green filter. These facts seemed to indicate that radiation of short wave-length was lethal to these organisms, and that, in all probability, the ultra-violet component of sunlight was the factor which was lethal.

The ultra-violet component of sunlight consists of the wave-lengths from 4000 A° to 2890 A°, as all shorter wave-lengths are absorbed by the atmosphere. The radiation of wave-length 2890 A° is extremely faint, and the shortest wave-length which could be detected at St. Andrews—by the use of a Hilger quartz spectrograph—was 2980 A°.

Apparatus

A three-compartment box, designed by the writer and constructed by R. D. Bradfield of the research workshop, Queen's University, was employed. This box was so constructed that the temperature in the three compartments would be uniform, and tests with three calibrated thermometers—one in each compartment—showed that this had been attained.

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Each compartment had a Corning glass filter as a window, and the energy values of these filters, as measured by a Moll-Richard-Gorczynski pyrheliometer were as follows:

Novial—82 per cent.
No. 980 (Total radiation)—91 per cent.
No. G986A (Ultra-violet)—14 per cent.

The Novial filter transmitted the visible spectrum, but no ultra-violet; the No. 980 transmitted both the visible spectrum and the ultra-violet; and the G986A transmitted only the ultra-violet, a very limited portion of the extreme violet, and a little of the extreme red, which latter was nearly all absorbed by the layer of water between the window and the tubes containing the experimental animals.

This three-compartment box, shown in Figs. 1 and 2, was set up on a specially built "light tower". The location for the light tower was so chosen that, by shifting the box, the experimental animals could receive direct sunlight from morning until sunset. The temperatures required for the various species were maintained by bringing the water which flowed through the box through a 30-ft. coil of ¼-inch copper tubing immersed in a barrel of ice and salt.

The animals experimented upon were placed in the quartz tubes shown in Fig. 2, and controls were kept in jars in the dark.

The data on the radiations from the sun, to which the animals used in these experiments were exposed, are given in gram calories per square centimetre per minute, as measured by a Moll-Richard-Gorczynski pyrheliometer. The readings given are, as far as possible, an average of the illumination received by the experimental animals while exposed to sunlight, but it must be realized that the passage of cumulus clouds across the sun—on days on which such clouds were present—prevents a precise average, and that the radiation of the early morning and the late evening—which was too weak to be measured by the pyrheliometer—may have had an influence on the results. These effects are unavoidable in experimental work in which natural radiation is employed, but it is believed that in spite of such fluctuations the results obtained are far more valuable, in interpreting natural conditions, than any experiments in which artificial illumination is employed.

As a tentative estimate, from work as yet uncompleted, it would seem as if the ultra-violet component of sunlight, at sea-level, latitude 45 deg. N., was about 2.5 per cent of the total radiation.

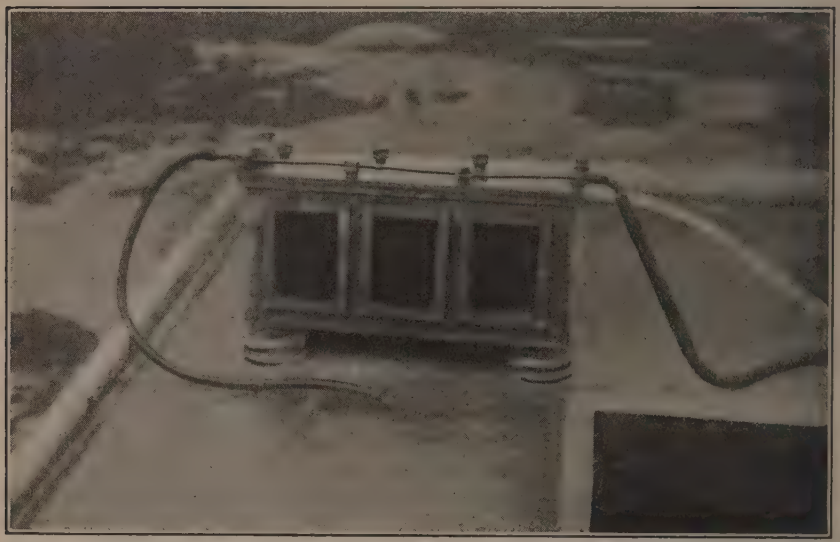

FIG. 1. *The Experimental Box, Closed. The rod across the top is a sprinkler, fed by the tube to the right, at the front. The water for the interior of the box comes in through the tube to the rear, and the exhaust is at the left.*

FIG. 2. *The Experimental Box, Open.*

Experimental

EXPERIMENTS ON *Calanus finmarchicus*.

Experiment No. 1.

July 21. Three female *Calanus finmarchicus* were placed in each tube behind each of the three filters at 11 a.m. Temperature, 8-10 deg. C. Total radiation (which will in the following records be abbreviated to "Py.", meaning pyrheliometer reading), 1.07 gm. cal.

July 22. Temperature, 10-12 deg. C.
Novial (i.e. under the Novial filter)—all normal.
Total (i.e. under the No. 980 filter)—2 dead, 1 weak.
U-V (i.e. under the U-V filter)—1 dead, 2 weak.

July 23. Temperature, 10-12 deg. C.
Novial—all normal.
Total—all dead.
U-V—2 dead, 1 weak.

July 24. No sunshine.

July 25.
Novial—all alive.
Total—all dead.
U-V—all dead.

Experiment No. 2.

July 25. Three *C. finmarchicus*, set in each tube as in Experiment 1, at 12 noon. Py., 1.00 gm. cal. for 4 hours.

July 26. 12 noon. Py., 0.97.
Novial—all normal.
Total—1 dead, 1 weak, 1 normal.
U-V—all normal.

July 26. 2 p.m. Py., 0.97.
Novial—all normal.
Total—all dead.
U-V—1 dead, 2 weak.

July 26. 4 p.m. Py., 0.70.
Novial—all normal.
Total—all dead.
U-V—all dead.

Experiment No. 3.

July 27. Three *C. finmarchicus* set in each tube as in Experiment 1, at 12 noon. Py., from 0.68 to 0.48.

July 28. Misty till 11 a.m. 11 to 1.30, Py., 0.87. Temperature 12.5 deg. C. 1.30 p.m.—all normal.

Cumulus clouds, cirro-stratus clouds and haze from 1.30 p.m. to 5.p.m. Py., from 0.85 to 0.50.

7.30 p.m. Temperature 12.5 deg. C. All normal.

July 29. 7.30 a.m. Temperature 12.5 deg. C.
Novial—all normal.
Total—1 dead, 2 weak.
U-V—all normal.

Sunshine from 9 to 10.30 a.m. Py., 0.90. From 10.30 a.m. to 12 noon dull. Faint sunlight from 12 to 1.30.

July 29. 1.30 p.m.
Novial—all normal.
Total—2 dead, 1 weak.
U-V—all normal.

July 29. 7 p.m.
Novial—all normal.
Total—all dead.
U-V—1 normal, 2 dead.

July 30. 12 noon. Py., 1.16 to 1.38.
Novial—all normal.
Total—all dead.
U-V—2 dead, 1 weak.

July 30. 1 p.m.
Novial—all normal.
Total—all dead.
U-V—all dead.

Experiments on *Aeginella longicornis*.

This is a caprillid which is found on weir-stakes below low-tide level.

Experiment No. 1.

July 30. Three *A. longicornis*, set as in the first experiment, at 10 a.m. Py., 1.38.

July 30. 1 p.m.
Novial—all normal.
Total—all restless and weak.
U-V—all weak.

July 30. 3 p.m. Py., 1.34. Temperature 12 deg. C.
Novial—all normal.
Total—all weak.
U-V—2 dead, 1 weak.

July 30. 4 p.m. Py., 1.24. 5 p.m. Py., 1.16.
Novial—all normal.
Total—all weak.
U-V—all dead.

July 31. 8 a.m.—all dead.

Experiment No. 2.

Aug. 3. Three *A. longicornis*, set at 12 noon. Py., 1.28.

2.30 p.m. Py., 1.23.
Novial—all normal.
Total—all normal.
U-V—all weak.

Aug. 3. 5.30 p.m.
Novial—all normal.
Total—all weak.
U-V—all dead.

Aug. 3. 7.00 p.m.
Novial—all normal.
Total—all dead.
U-V—all dead.

Experiments in hatching embryos of *Lophius piscatorius*.

In this species of fish the eggs float at the surface, and the embryos develop, and finally become free-swimming, while exposed to the full radiation of the sun. There is no need to give, in detail, the record of these experiments, as the hatching embryos of this species remained equally active under all the filters from Aug. 3 until Aug. 8, when the experiment was discontinued.

Experiments on the hydroid *Tubullaria crocea*.

This species occurs only in situations at some considerable depth below low-tide level.

Experiment No. 1.

Aug. 3. Py., 1.28. Temperature, 15 deg. C. One *T. crocea* set in each tube at 12 noon.

Aug. 3. 2.30 p.m. Py., 1.23—all normal.

Aug. 3. 7.00 p.m.
Novial—all normal.
Total—all dead.
U-V—all dead.

Experiment No. 2.

Aug. 4. Py., 1.21. Temperature, 15 deg. C. Three *T. crocea*, set in each tube, at 12 noon.

Aug. 4. 5.30 p.m. Py., 1.09. Temperature 15 deg. C.—All normal.

Aug. 5. 9.00 a.m. Foggy.
Novial—1 dead, 2 weak.
Total—all dead.
U-V—all dead.

Experiment No. 3.

Aug. 6. 1 p.m. Py., 1.13. Temperature, 15 deg. C. One *T. crocea* set in each tube at 1 p.m.

Aug. 6. 4.00 p.m.
Novial—normal.
Total—normal.
U-V—dead.

Aug. 6. 7.00 p.m.
Novial— normal.
Total—dead.
U-V—dead.

EXPERIMENTS ON *Tortanus discaudatus*.

This species of copepod comes to the surface only when the light is faint.

Experiment No. 1.

Aug. 10. 10.30 a.m. Py., 1.26. Temperature, 15 deg. C. Three *T. discaudatus* (female) set in each tube.

Aug. 10. 3.00 p.m. Py., 1.22.
Novial—all normal.
Total—all dead.
U-V—2 dead, 1 weak.

Experiment No. 2.

Aug. 11. 10.30 a.m. Py., 1.26 to 1.30. Three *T. discaudatus* (female) set in each tube.

Aug. 11. 1.30 p.m. Temperature, 12 deg. C.
Novial—all normal.
Total—all normal.
U-V—1 dead.

Aug. 11. 5.30 p.m. Py., 1.03. Temperature, 12 deg. C.
Novial—all normal.
Total—2 dead.
U-V—1 dead.

Aug. 12. 10.00 a.m.
Novial—1 dead.
Total—2 dead.
U-V—all dead.

Experiment No. 3.

Aug. 18. 10.00 a.m. Sunshine, but great fluctuations due to clouds. Three *T. discaudatus* (female) set in each tube.

Aug. 18. 2.00 p.m. Temperature, 12 deg. C.
All normal.

Aug. 18. 5.30 p.m. Temperature, 15 deg. C.
Novial—all normal.
Total—2 dead.
U-V—all normal.

Aug. 19. 9.00 a.m.
Novial—all normal.
Total—all dead.
U-V—all dead.

EXPERIMENTS ON *Eurytemora herdmani*, MALE.

Experiment No. 1.

Aug. 10. 10.30 a.m. Py., 1.26. Temperature 15 deg. C. Three *E. herdmani* set in each tube.

Aug. 10. 3.00 p.m. Py., 1.22. Temperature, 15 deg. C.
Novial—2 normal, 1 dead.
Total—all dead.
U-V—all dead.

Experiment No. 2.

Aug. 10. 5 p.m. Py., 1.02. Three *E. herdmani* set in each tube.

Aug. 11. 10.30 a.m. Py., 1.26. Temperature, 12 deg. C.

Aug. 11. 1.30 p.m. Py., 1.30.
Novial—all normal.
Total—2 dead.
U-V—1 dead.

Aug. 11. 5.30 p.m. Py., 1.03.
Novial—all normal.
Total—all dead.
U-V—2 dead.

Aug. 12. 9.00 a.m. Temperature, 13 deg. C.
Novial—all normal.
Total—all dead.
U-V—all dead.

Experiment No. 3.

Aug. 18. 10 a.m. Py., 1.00. Temperature, 15 deg. C. Three *E. herdmani* set in each tube.

Aug. 18. 2.00 p.m. Py., 1.00.
Novial—all normal.
Total—all dead.
U-V—2 dead, 1 normal.

Aug. 18. 5.30 p.m. (Faint sun since 2.30 p.m.).
Novial—all normal.
Total—all dead.
U.V—all dead.

Experiments on *Acartia clausi*.

This copepod lives at the surface of the sea.

Experiment No. 1.

Aug. 20. 2.00 p.m. Py., 1.13. Six *A. clausi* set in each tube.
6 p.m.—all normal.

Aug. 21. 10 a.m.—all normal. Bright sun all day.

Aug. 21. 7 p.m.—all normal.

Aug. 22. 10.00 a.m. Py., 1.21. All normal.

Aug. 22. 2.30 p.m. Py., 0.97.
Novial—all dead.
Total—all dead.
U-V—4 dead.

Experiment No. 2.

Aug. 22. 3.00 p.m. Py., 1.00. Temperature, 14 deg. C. Eight *A. clausi* set in each tube.

Aug. 23. 2.00 p.m. (Fog since morning).
Novial—all dead.
Total—all dead.
U-V—5 dead.

Experiment No. 3.

Aug. 25. 10.30 a.m. Py., 1.42. Temperature, 15 deg C. Six *A. clausi* set in each tube.

Aug. 25. 7.30 p.m.—all normal.

Aug. 26. 10.00 a.m. Py., 1.30., Temperature, 15 deg. C.
Novial—4 dead.
Total—all dead.
U-V—all dead.

Acknowledgment

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